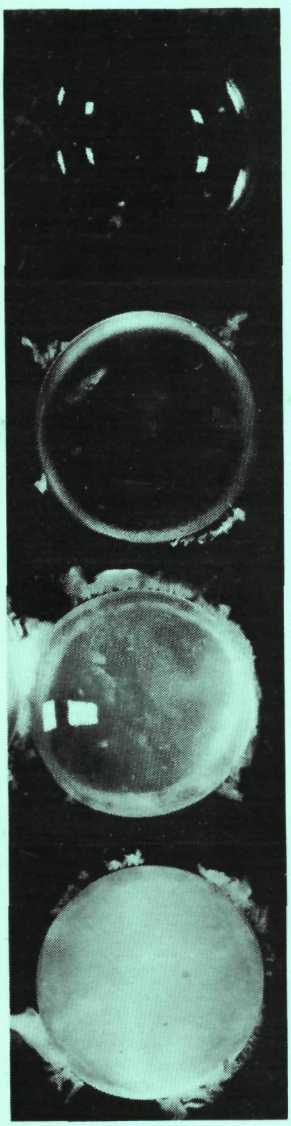
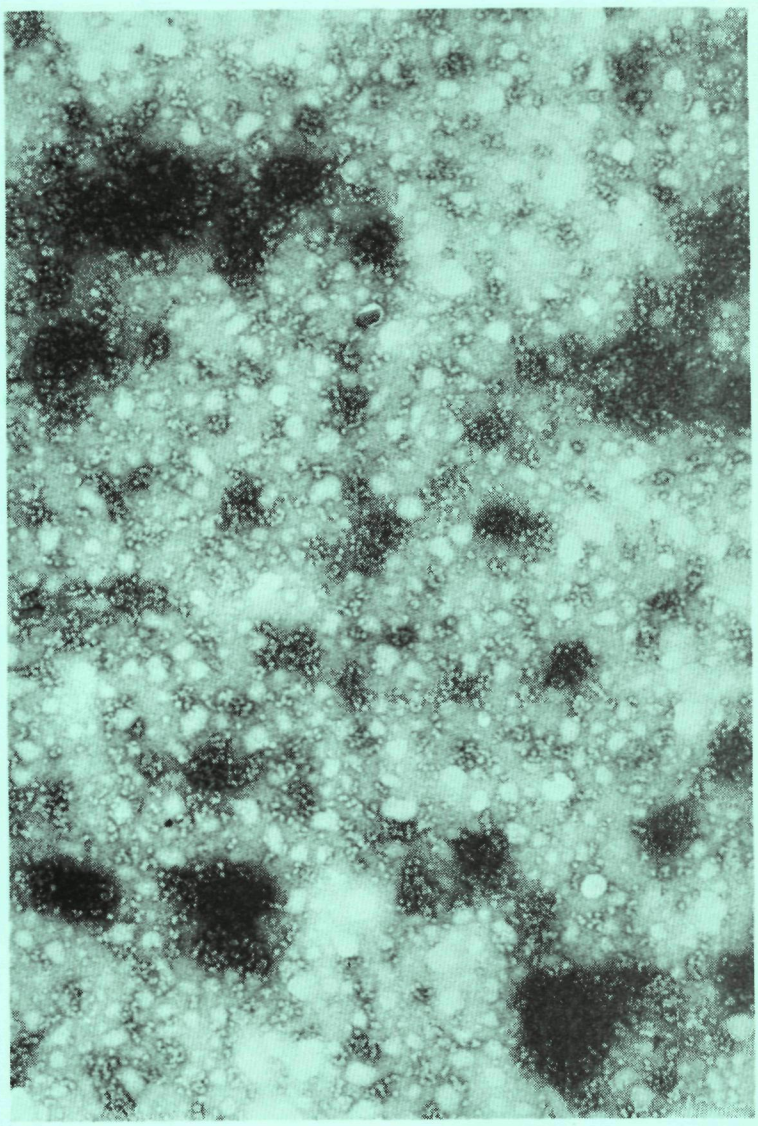
THE STRUCTURAL PROTEINS OF THE RABBIT EYE LENS AFTER X-IRRADIATION





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TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE
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Nijmegen

Aan mijn ouders

Aan mijn man

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Chapter 1

The Eye Lens

Normal lens

Dimensions

The vertebrate lens is a transparent, avascular, semi-solid body having a biconvex shape. In the three-month-old rabbit lens, the central axis from anterior to posterior pole is about 7 mm in diameter, while the equatorial diameter is about 10 mm. The weight of a three-month-old rabbit lens is approximately 300 mg. The average dimensions of a mature human lens are 4 and 10 mm for its thickness and equatorial diameter, respectively. The lens wet weight is approximately 200 mg.

Chemistry

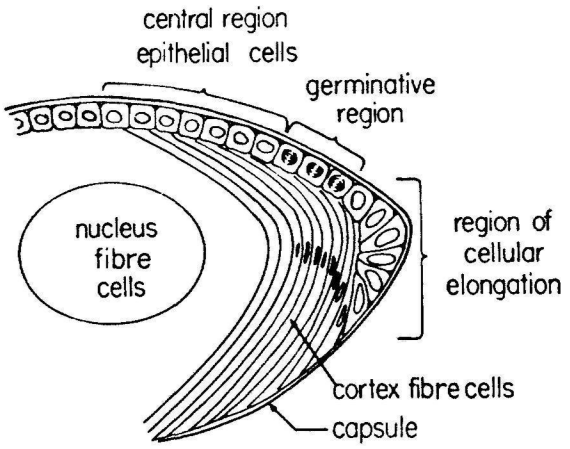
Chemically, the lens essentially consists of protein and water. The protein is very abundant, forming approximately 35% of the total wet weight. Mörner (1894) divided the proteins from bovine lenses into 2 fractions: soluble and insoluble protein (albumoid). The soluble protein was further divided into three fractions, now called α-, β- and γ-crystallin. Krause (1932 and 1934) estimated that albumoid comprises about 12.5%, α-crystallin 31.7%, β-crystallin 53.4% and γ-crystallin 1.5% of the total protein. In addition he found minor amounts of glyco- and nucleoprotein. Some of the properties (amongst others molecular weight, electrophoretic mobility, thiol content, N-terminal amino groups) of the crystallins have been determined (for a detailed report see Waley, 1969); especially α-crystallin was investigated throughly (reviewed by Bloemendal, 1972).

Earlier papers reported that α-crystallin consists of four subunits, namely two acidic and two basic subunits (Schoenmakers and Bloemendal, 1968; Björk, 1968). Recently it has been shown that only cortical α-crystallin has four subunits, whereas nuclear α-crystallin reveals a more complex subunit structure (van Kleef, Nijzink-Maas and Hoenders, 1974). The amino-acid sequence of the polypeptide chains αA_2 and αB_2 have been elucidated (van der Ouderaa, de Jong and Bloemendal, 1973 and van der Ouderaa, Hilderink, de Jong and Bloemendal, 1974). Since some years the existence of high molecular weight proteins is known (Spector, Li, Augusteyn, Schneider and Freund, 1971). Their polypeptide chain composition is similar to that of α-crystallin.

Mörner (1894) was the first who observed that the water-insoluble albumoid is mainly found in the nucleus (older part) of the lens. The decreasing amount of α-crystallin and the increasing amount of albumoid found, going from the outer to the inner part of the lens, led to the suggestion that α-crystallin was being converted into albumoid *in vivo* (Krause, 1934). The close relationship between α-crystallin and albumoid was evidenced by applying electrophoretical methods, immunochemical and amino-acid analysis (see Waley, 1969). The decrease in soluble and increase of insoluble proteins seems to be a function of age.

Morphology

The lens is built up of three parts: the capsule, the epithelium and the fibre cells. The capsule is a tough, clear membrane, entirely surrounding the rest of the lens. The epithelium extends as a monolayer underneath the anterior capsule. There are no epithelial cells on the posterior subcapsular surface of the lens. The lens fibre cells originate from the lens epithelium. In a zone just anterior to the equator, which is



Schematic drawing of part of the lens. (From Papaconstatinou, J.: Molecular aspects of lens cell differentiation, Science **156**, 338, 1967)

called the germinative region, the epithelial cells divide at a high rate. At the equator these cells are elongating to form fibre cells. This process continues until the end of life, although the rate decreases. The lens thus contains essentially one type of cells, all formed in the germinative region. The outer layers (cortex) represent the newer part of the lens and the central region (nucleus) the older part. There is no mechanism known whereby the lens can rid itself, on a cytological scale, of abnormal, damaged or non-viable cells. So it is a unique tissue, as the entire history of its continuous growth is preserved in it.

Function

The sole function of the lens seems to be the refraction of image-bearing light beam in a controllable fashion. It is essential, therefore, that the lens maintains its transparency. Proper function may fail, for instance, if it becomes opaque.

Cataractous lens

General considerations

The opacification of the lens resulting in reduction or loss of vision is called cataract. The etiology of cataract is complex. Degenerative changes accompanying aging processes, intraocular disease, genetic, metabolic, endocrine, chemical, radiation

and traumatic disorders may be causes of cataract formation. According to Sorsby (1962) each year some 1.2 million people throughout the world develop visual impairment due to cataract. What we know now is that the clinical opaque appearance of the cataractous lens is the result of three kinds of disturbances, namely: the presence of very high molecular weight crystallins, accumulation of irreversibly precipitated lens protein and of the numerous irregular zones with different refractive indices caused by water clefts. All three types of disturbances scatter light.

Cure

Up till now the only cure for cataract is removal by surgery of the diseased lens. It would be most desirable to develop alternative methods of treating cataract without resorting to surgery and methods of preventing or retarding the development of this disorder.

Problems in research

A problem of practical importance is that the availability of normal human lenses is minimal. In order to ultimately understand the mechanisms leading to cataract it is necessary to study the very early changes taking place in cataract formation, and such lenses with beginning cataract are as a matter of course not extracted. So, one must turn to animal models. However, it is often hazardous to extrapolate observations made on the experimental animal to an apparently similar disease in man. Nevertheless, it appears highly likely that cataractous changes are very similar, irrespectively they occur in man or animals, for the following reasons: in various and diverse species the lens shows organ rather than species specifity, its avascularity and mode of growth which is analogous and the marked similarity of the metabolism.

The next problem is to choose an experimental cataract which most resembles the senile cataract. Cataract in animals can be induced by chemical means, amongst others by feeding them naphtalene or sugars (galactose, xylose or glucose) or by irradiations, e.g. with α-, β-, γ- or X-rays. The X-ray induced cataract seems to be the most eligible because X-rays produce cataract which resembles senile cataracts (Kuck, 1970; Lerman and Zigman, 1963; Dische, Elliot, Pearson and Merriam, 1959). Another advantage is that only one eye is exposed, while the contralateral eye can serve as a control.

Experimental X-ray cataract in rabbit lens

Extensive research on experimental cataract induced by X-rays has been done since Chalupecky (1897) observed that lenses from rabbits were sensitive for this radiation. Aulamo (1931) had succeeded to induce cataract experimentally in rabbit lenses by X-rays. Numerous clinical observations were made and described amongst others by Rohrschneider (1929). It was observed that there is a latency period between the moment of irradiation and the onset of cataract. This latency period is inversely related to the dose of X-rays. There is a similar relationship between the susceptibility of the lens and the age of the animal: the younger the lens the more vulnerable to X-ray damage.

Morphologic and histochemical investigations have shown that the initial changes in such lenses occur mainly in the epithelial layer. Experiments in which specific

portions of the lens were shielded indicate that the achievely growing equatorial part (germinative zone) is most sensitive to injury by X-ray (Goldmann and Liechti, 1938; Alter and Leinfelder, 1953; Richards, Riley and Leinfelder, 1956; Pirie and Drance, 1959). Von Sallmann and others (1950, 1951, 1952, 1955, 1957 and 1959) have demonstrated that early radiation damage precedes the development of clinically visible changes by many days or weeks. They noted the rapid and marked inhibition of cellular mitosis which soon occurs after exposure. Furthermore, shortly thereafter, nuclear fragmentation, degeneration and extrusion of chromatin material from these cells became manifest. The initial and marked depression of cell division was followed by a relatively short-lived but marked increase in mitosis. In rabbit this "overshoot" occurred from approximately 7 to 20 days after exposure with 2000 Rad.

Von Sallmann and Locke (1951) suggested that the pathogenesis of X-ray cataract might be explained by postulating two sets of lesions. The primary lesion would be located in the nuclei of the germinative cells, while the secondary stage, which leads to the destruction of the lens fibre cells and eventual dense opacification, could be due to chemical changes.

Biochemical results in the form of direct inactivation of key enzymes or metabolic sequences arising as a result of suppression of mitosis, has been reported but no conclusive evidence was obtained. Despite the lack of unequivocal data the changes observed as a result of radiation will be described.

One of the early chemical changes observed in the lens following exposure is a rapid fall in the level of the sulfhydryl groups (Pirie, van Heyningen and Boag, 1953; van Heyningen, Pirie and Boag, 1954; Nordmann and Mandel, 1955; Pirie, van Heyningen and Flanders, 1955; Hockwin, 1962; Oreckhovich, Firvarova, Kedrova and Ledikova, 1962). The most marked decline occurs in the concentrations of gluthathione. This decrease began before any clinical abnormality could be seen. A decrease in the protein SH occurred only when opacification had begun and it was always less pronounced than the decrease in gluthathione. Similarly the activity of those enzymes which require SH-groups is diminished prior to the enzymes which are not SH-dependent.

Another metabolic change is a decrease in oxygen consumption and phosphate uptake (Hockwin and Bergeder, 1958). The levels of the adenine nucleotides were essentially unaffected by radiation (Hockwin, 1962).

In the early stages of opacity formation the irradiated lens fails to gain weight as fast as its normal fellow (van Heyningen, Pirie and Boag, 1954). The early failure of growth may well be due to a failure in protein synthesis. However, no early changes were found on the electrophoretic behavior of the soluble proteins of the exposed lens (Rupe, Monsul and Koenig, 1958).

It has been widely held that the biological action of ionizing radiation is based partly or entirely on alterations in the permeability of cell membranes. Von Sallmann and Locke (1951) investigated it by using radioactive tracers. They found that alteration in permeability occurred only after opacification had begun. So they concluded that this was not a factor in the development of cataract, rather, that it might possibly influence the progress of opacification.

On the basis of extensive biochemical investigations, Pirie (1956), confirming von Sallmann (1951), concluded that the development of radiation cataracts may proceed in two stages: the first is due to direct effects on the lens epithelium while the second stage involves damage to the lens fibre cells.

Human senile cataract

Senile cataract in man can be classified generally in cortical and nuclear cataract Cortical cataracts are clinically identified by the early appearance of vacuoles, which in a later stage become water clefts, furthermore opacities occurring in a variety of shapes are found in the cortical layer of the lens Nuclear cataracts are characterized by the hardening (sclerosis), opacification and coloration of the inner part of the lens Occasionally the lens nucleus is opaque but still soft in consistency.

Morphologically, classification is no problem because the location of the opacities under study can be pinpointed. Still, relatively few electron microscopic studies are reported (a.o. Cohen, 1965, Kobayashi, 1969, Hogan, Alvarado and Weddell, 1971 and Yamada and Shikano, 1973).

The great diversity of types in senile cataracts within the two main categories posed a problem for biochemical studies No two cataracts are alike. There are differences in age of the lenses, differences in location and form of the opacities and also differences in color If one has overcome all these difficulties and succeeded in pooling similar types of cataractous lenses, then the question arises whether one is working exact enough, since some portion of the diseased lens is often clear and possibly still healthy. Working with such a mixture of affected and normal tissue can obscure possible differences Such difficulties are among the causes for the different results found by many authors.

A brief description of the work done in the past decades is given here. For detailed accounts the reader may be referred to articles of Lerman, 1964, Duke-Elder, 1969, Waley 1969, van Heyningen, 1969, Kuck, 1970, Nordmann, 1972 and Barber, 1972, 1973.

A decrease in lens volume and lens weight in senile supranuclear (cortical) cataract was found by Bellows (1944), Nordmann (1954), Barber (1968), Delmarcelle and Luyckx-Bacus (1971) and Maraini and Pescatori (1972) For nuclear cataract no general conclusion concerning its lens volume and lens weight could be reached, since different results were obtained by many authors. Some observed an increase, others say that it is normal.

Enzyme activities in the different pathways of carbohydrate metabolism have been determined separately for each type of cataract (Hockwin and Berghoff, 1966, Friedburg, 1966, 1967, 1972, Maraini, Santori and Carta, 1967, Friedburg and Meyer, 1968 and Cioli, 1972) Here the various forms of cataract have given quite different results. A decrease in gluthathione is also observed.

In cortical cataract a decrease in total protein is established (Cahn, 1881, Maraini and Pescatori, 1972). In senile nuclear cataract the total protein content seemed to be normal (Maione and Maraini, 1969, Sheridan and Zigman, 1971 and Maraini and Pescatori, 1972) A feature of advanced cataract is loss of soluble protein, preferentially of the smaller molecules (Salit, 1936, Mach, 1963, Santori and Carta, 1967, Charlton and van Heyningen, 1968, Malik, Gupta, Chatterji and Agarwal, 1969, François, Rabaey and Boyen-Rikkers, 1969, Sheridan and Zigman, 1971, Maraini and Pescatori, 1972).

Other changes of the soluble protein in human cataract are conformation changes, oxidation of SH-groups (Harding, 1970, 1972, Auricchio and Testa, 1972), photo-oxidative cross-linking and pigmentation (Pirie, 1968, Clark, Zigman and Lerman, 1969, Auricchio and Testa, 1972, Pirie, 1972 and Grover and Zigman, 1972) These changes may coexist to variable degrees in morphologically different cataracts

and possibly result in insolubilization of lens protein. Furthermore, opacification may be caused by aggregation to larger, light-scattering particles (Benedek, 1971 and Jedziniak et al., 1973). Another plausible cause for opacification is the light scattering due to steep refractive index gradients between regions of high and low protein concentrations. This phenomenon accompanies swelling of the cataractous lens, and is probably initiated by changes in the permeability of cell membranes or by escessive accumulation of metabolites (Salit, 1938; Duncan and Croghan, 1969; Kinoshita, 1965 and Pirie, 1968). According to Barber (1973) this pathological swelling is a more probable mechanism for producing opacification than alterations of soluble protein. However, Maraini and Mangili (1973) postulated that pathological swelling is a feature characteristic for cortical cataract, whereas alterations of the lens proteins are more specific for nuclear cataract.

Aim of the present study

In cataract, as a result of faulty biosynthesis or metabolic errors, changes in the proportions and nature of the polypeptides making up the crystallins might occur. These changes could lead to extensive aggregation and insolubilization.

It was our aim to detect possible qualitative and quantitative differences in the distribution and subunit structure of the protein fraction from normal and X-ray induced cataractous rabbit lenses. The experience and results obtained should further be applied to the study of human senile cataractous lens.

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Characterization of the Soluble Proteins from Rabbit Eye Lens

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Fractionation of the soluble proteins of rabbit lens by gel chromatography on Sepharose 6B revealed six components, viz. HM-, α-, β_1-, β_2-, β_3- and γ-crystallin. On isoelectric focusing α-crystallin revealed a diffuse region, whilst approximately 10 bands were found for the β-crystallins and 7–8 components for γ-crystallin. Immunoelectrophoretically HM-crystallin revealed two precipitin lines, one in the α- and one in the β-mobility range. Sedimentation coefficients of 18·0, 8·0, 3·7, 2·2 and 2·1 S were calculated for α-, β_1-, β_2-, β_3- and γ-crystallin, respectively. By isoelectric focusing in the presence of 6 M-urea, 13 bands between pH 5·3 and 6·9 were found for HM-crystallin, approximately six bands between pH 5·6 and 6·9 for α-crystallin, about 13 bands between pH 5·9 and 7·0 for the β-crystallins and 7–8 major bands between pH 7·0 and 7·8 for γ-crystallin. Molecular weight estimation by SDS gel electrophoresis revealed values between 16 000 and 39 000 for HM-crystallin and the β-crystallins, whereas values of approximately 21 000 and 20 500 were found for α- and γ-crystallin.

1. Introduction

Investigation of the protein composition of the rabbit eye lens by chemical means was started in 1954 by François, Wieme, Rabaey and Neetens. These authors reported three protein bands using paper electrophoresis. In 1959 Wood, Massi and Solomon applied continuous flow electrophoresis on rabbit lens extract and obtained five components. Mason and Hines (1966), using the same separation technique, found three components, viz. α-, β- and γ-crystallin. Björk (1968) compared α-crystallin preparations from several species, including rabbit, by polyacrylamide gel electrophoresis.

Immunochemical analyses of eye lens proteins from several species were performed by Halbert et al. (1957) and Manski, Auerbach and Halbert (1960). In their studies the rabbit lens was included. Cross-reactions of lens proteins of several species (organ specificity) appear to be due to similarity of varying numbers of these antigens. Björk (1968) found identical antigenic sites for α-crystallins from several mammalian species including that from rabbit.

In our laboratory studies on possible changes in polypeptide chain composition of crystallins in X-ray induced cataract in rabbit lenses are in progress. The first part of this study comprised the characterization of normal rabbit lens proteins with the newest available separation and analytical techniques. This paper reports on results obtained by application of gel chromatography on agarose, isoelectric focusing on polyacrylamide gels in the presence and absence of urea, molecular weight estimation in SDS-polyacrylamide gels, sedimentation analysis and immunochemical methods. Calf lens extract was used to compare the results with well-known data.

2. Materials and Methods

Extraction of water-soluble lens proteins

Three- to six-month-old white New Zealand rabbits were used. The lenses were carefully removed and freed from vitreous, aqueous and capsular material. The fresh lenses were

homogenized in a porcelain mortar Each lens was extracted three times in 1 ml of 0·9% saline solution containing 0·015 M-phosphate, pH 7·3. The pooled extracts were centrifuged at 18 000 rev/min in a SS 34 rotor for 20 min at 4°C in a Sorvall centrifuge The supernatant solutions were pooled and then dialyzed against fresh demineralized water The salt free protein solutions were lyophilized Lenses from approximately 14-week-old calves were extracted in the same way.

Gel chromatography

Three hundred milligrams of dry protein were dissolved in 3·0 ml of saline–phosphate solution pH 7·3. Sepharose 6B (exclusion limit $4{\cdot}10^6$ Daltons) was used in columns with an internal diameter of 3 cm and a length of 120 cm. The elution buffer was the same saline–phosphate solution The flow rate was controlled by a Cenco peristaltic pump at 30 ml/hr. Fractions of 6 ml were collected. The fractions were combined in an appropriate manner, dialyzed against distilled water and lyophilized.

Sedimentation analysis

Sedimentation coefficients were determined with a Spinco E analytical ultracentrifuge using u v absorption optics at 280 nm in combination with an automatic scanner. Protein samples were dissolved in phosphate buffered saline at concentrations corresponding to $A_{280}^{1\,cm}$ = about 1·0 Sedimentation coefficients were corrected to water at 20°C ($s_{20,w}$).

Immunochemical procedures

Antibodies to total water-soluble calf lens proteins were isolated from hyperimmunized white New Zealand rabbits. Double-diffusion precipitin tests were performed by the method of Ouchterlony (1958) Immunoelectrophoresis was carried out according to the micro-modification of Scheidegger (1955) using LKB apparatus.

Isoelectric focusing on thin-layer polyacrylamide gels

The method according to Bours (1971) was used. Isoelectric focusing was performed on polyacrylamide slabs containing 0·5% Ampholine pH 3–10 Sample solutions containing 2% protein were applied.

Gel electrophoresis in the presence of sodium dodecyl sulphate (SDS)

For the determination of molecular weight polyacrylamide gels containing 0·1% SDS according to Laemmli (1970) were used. To the upper buffer and to the sample solution 0·04% DTT* was added. The current used was 3 mA/gel. The electrophoretic run took 2·5 hr. For calibration bovine serum albumin, egg albumin, chymotrypsinogen A, myoglobin and cytochrome C were used.

Isoelectric focusing in urea containing polyacrylamide gels

Isofocusing was done according to Wrigley (1968) in the presence of Ampholine pH 5–8 in 6 M-urea. To the Ampholine layer and the sample 0·04% DTT was added Runs were performed in 15 cm tubes for 5 hr at 350 V after a pre-run of 1 hr to remove oxidizing agents.

3. Results

Gel chromatography

A protein fraction eluted with the void volume of the Sepharose 6B column was designated as HM-crystallin (high-molecular weight crystallin). It was present in

* Dithiothreitol.

rabbit as well as in calf lens extract. After this HM fraction 4–5 protein fractions were obtained (Fig. 1), comprising the α-, β- and γ-crystallins as identified by several methods. The elution pattern of rabbit eye lens extract is essentially the same as that of bovine lens. Slight differences are found in the β-region. Rabbit lens extract showed three β-crystallin fractions, β_1, β_2 and β_3, whereas calf lens extract revealed two β-crystallins, β_H and β_L (Hoenders, van Kamp, Liem-The and van Kleef, 1973).

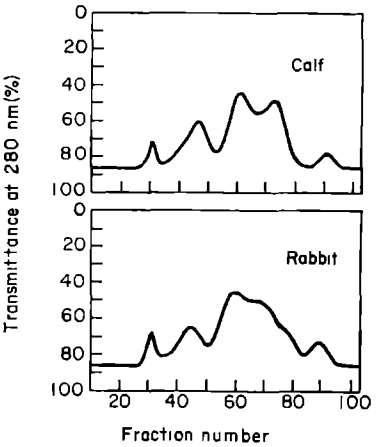

FIG. 1. Gel filtration of water-soluble rabbit and calf lens proteins on Sepharose 6B.

Sedimentation analysis

The sedimentation coefficients of fractions with the highest concentration of the various crystallins obtained by gel chromatography on Sepharose 6B are presented in Table 1. A sedimentation coefficient of HM-crystallin could not be calculated because no sharp boundary was obtained.

TABLE I

Sedimentation coefficients of lens crystallins, s_{20w},(S)

	α	β_1	β_2	β_3	γ
Rabbit	18·0±0·3	8·0±0·2	3·7±0·1	2·2±0·2	2·1±0·2
	α	β_H	β_L		γ
Calf	17·6±0·6	8·1±0·3	3·5±0·2		2·3±0·2

Averaged values of 2–3 sedimentation velocity runs are included in the table.

Immunochemical analysis

Immunodiffusion using anti-calf lens serum against rabbit and calf lens extract revealed two cross-reacting antigens. In addition, four to five calf lens antigens and two rabbit lens antigens which did not cross-react, were observed [Fig. 2(a)]. Immunoelectrophoresis showed arcs in the α-, β- and γ-crystallin mobility ranges for the water-soluble rabbit and calf lens proteins [Fig. 2(b)]. A similar overall pattern for rabbit

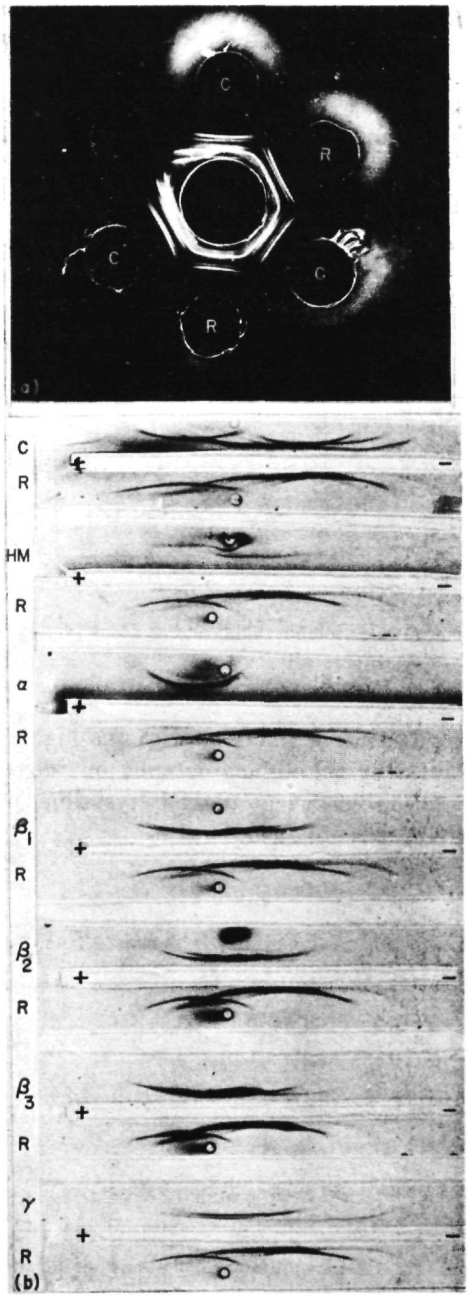

FIG 2. (a) Agar precipitin test against anti-calf lens serum. Antigens were rabbit lens extract (R) and calf lens extract (C). (b) Immunoelectrophoretic reactions of rabbit lens extract (R) and calf lens extract (C) as well as reactions of rabbit lens crystallins (HM, α, β_1, β_2, β_3 and γ) against anti-calf serum. Per hole 2 μl of 5% protein solution was applied. Electrophoresis was performed at 6 V/cm and 4 mA per slide for 3 hr. The gels were stained for 15 min in 0·1% Amido black dissolved in 2% acetic acid. Destaining was performed in 2% acetic acid to which 2% glycerol was added.

and bovine total lens extract was found. For HM-crystallin from rabbit lens we found two precipitin lines, one in the α- and one in the β-crystallin mobility range. Rabbit β_1-, β_2- and β_3-crystallins revealed precipitin lines in the β-mobility range. γ-Crystallin showed precipitin arcs in the γ- and β-mobility range.

Isoelectric focusing on thin-layer plates

The isoelectric focusing pattern of the rabbit and bovine lens preparations showed much similarity (Fig. 3). HM-crystallin could not be dissolved in demineralized water and, therefore, no pattern could be obtained. α-Crystallin revealed a diffuse zone for rabbit and calf lens around its isoelectric point. The β-crystallins showed complex patterns with approximately 10 bands for rabbit and bovine lens. γ-Crystallin revealed 7–8 components.

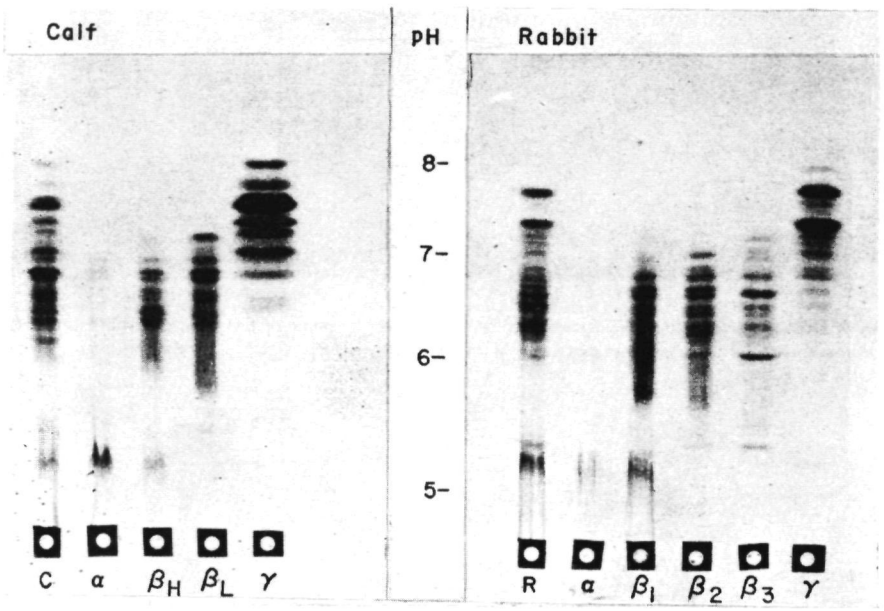

FIG. 3. Isoelectric focusing on thin-layer polyacrylamide gels of water-soluble rabbit and calf lens crystallins. From left to right gel patterns of calf lens extract (C), α-, β_H-, β_L-, γ-crystallin and of rabbit lens extract (R), α-, β_1-, β_2-, β_3-, γ-crystallin.

Isoelectric focusing in the presence of 6 M*-urea*

The resolution of subunits by isofocusing is markedly improved compared with gel electrophoresis in 6 M-urea. HM-crystallin revealed 13 bands between pH 5·3 and 6·9, α-crystallin approximately six bands between pH 5·6 and 6·9, the β-crystallins about 13 bands between pH 5·9 and 7·0 and γ-crystallin 7–8 major bands between pH 7·0 and 7·8 (Fig. 4). HM-crystallin shows two bands in the pH 5·3–5·5 range which are absent in the α-crystallin pattern (bands 1 and 2 of HM-crystallin, Fig. 4). In the pH range of 5·9–6·9 bands 6, 7, 8, 9, 11 and 12 of HM-crystallin were not seen in the α-crystallin pattern, whereas bands 3, 5 and 13 have the same isoelectric points as αA_1, αA_2 and αB_2, respectively. It is noteworthy that αB_1 is lacking and αB_2 is very faint. Two other bands, designated as αA_X (band 4) and αB_X (band 10) were

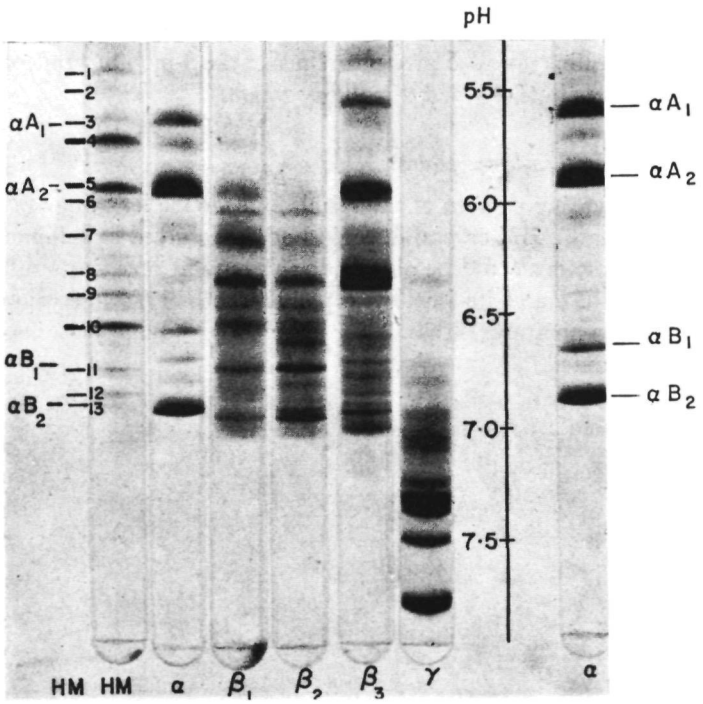

FIG. 4. Isoelectric focusing on polyacrylamide gels in the presence of 6 M-urea of rabbit and calf lens crystallins. From left to right gel patterns of rabbit HM-, α-, β_1-, β_2-, β_3-, γ-crystallin and of calf α-crystallin are shown.

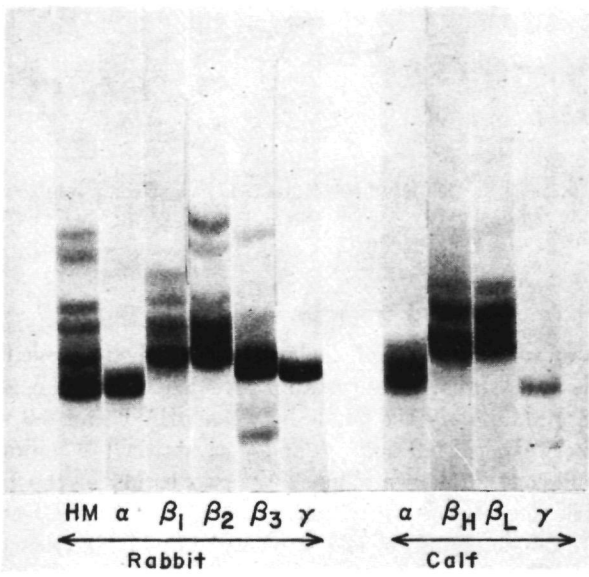

FIG. 5. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. From left to right gel patterns of rabbit HM-, α-, β_1-, β_2-, β_3-, γ-, and of calf α-crystallin are shown.

seen in HM- as well as in α-crystallin. Furthermore bands 7, 8, 11 and 12 have positions corresponding with β-crystallin bands. β_1- and β_2-crystallin revealed the same pattern with only some quantitative differences. β_3-Crystallin, on the other hand, has a rather different pattern. Like HM-crystallin it has bands in the acid region (about pH 5·5).

Gel electrophoresis in the presence of SDS

HM-crystallin revealed a pattern with eight bands ranging from 16 500–39 000 Daltons (Table II and Fig. 5). Two bands, corresponding with 16 500 and 17 500 Daltons, are very faint and not visible in Fig. 5. The heavy band of approximately 21 000 corresponds with the α-crystallin polypeptide chains. Applying smaller amounts, this band is split up into two separate bands, namely that of the αA and αB chains. β_1-Crystallin revealed four bands with molecular weights between 23 000 and 32 000, β_2-crystallin five bands between 23 000 and 39 000. β_3-Crystallin showed a pattern with five bands corresponding to molecular weights between 16 000 and 36 500. Finally, γ-crystallin revealed only one band corresponding to a molecular weight of approximately 20 500.

TABLE II

Molecular weights of polypeptides from lens crystallins, estimated by gel electrophoresis in the presence of sodium dodecyl sulphate

Rabbit	HM	39 000	36 000		28 500	26 000	23 000		21 000*	(17 500)	(16 500)
	α								21 000*		
	β_1			32 000	28 500	26 500	23 000				
	β_2	39 000	36 000		28 500	26 500	23 000				
	β_3		36 500			26 500		22 000		17 700	16 000
	γ								20 500		
Calf	α							22 000	20 000		
	β_H		(36 000)		28 500	26 000	23 000				
	β_L		(36 000)		28 500	26 000	23 000				
	γ								21 000		

* The heavy band of 21 000 daltons actually represents two bands, the α_A and α_B band.
Values of minor components are given in parentheses

4. Discussion

It appears that a satisfactory fractionation of the water-soluble rabbit lens proteins is possible by gel chromatography on Sepharose 6 B. Using this method the soluble proteins were separated into six components (Fig. 1). Mason and Hines (1966) found three components by gel filtration on Sephadex G75 and continuous-flow electrophoresis. Wood, Massi and Solomon (1959), also using continuous-flow electrophoresis but under different experimental conditions, obtained five components. Halbert et al. (1957) found five soluble antigens in the rabbit lens in their immunological studies.

The sedimentation coefficients (Table I) of a α-crystallin (18·0 S) and γ-crystallin (2·1 S) from rabbit lens are in fair agreement with the values found by Mason and Hines (1966), viz. 18·9 S and 2·5 S. Furthermore the ultracentrifugal data of rabbit α- and γ-crystallin agree closely with those found for bovine α-crystallin (Bloemendal, Bont, Jongkind and Wisse, 1964; Papaconstantinou, Resnik, Saito 1962; Spector, 1964; Björk, 1963, 1964a) and bovine γ-crystallin (Björk, 1961, 1964c; Spector, 1964).

On isoelectric focusing α-crystallin gave one diffuse band around its isoelectric point (Fig. 3). The β-crystallins showed complex patterns which indicates that they are composed of numerous proteins. This is in general agreement with Björk (1964b) who found four main fractions by gel filtration of β-crystallin from calf lens, each fraction containing some additional minor components. The β_1- and β_2-crystallins gave similar patterns with only quantitative differences. The β_3-crystallin fraction, however, showed a different pattern characterized by two heavy bands in the region between pH 6·0 and 7·0 γ-Crystallin showed 7–8 components on isoelectrofocusing in the absence and presence of urea (Figs. 3 and 4). This is in agreement with results of Hines and Olive (1970), who found eight components in γ-crystallin of rabbit by column chromatography on CM-cellulose. These authors concluded that γ-crystallin of rabbit is similar to that of calf lens.

Fractionation of the crystallin subunits by electrophoresis on polyacrylamide gels in the presence of 6 M-urea was not satisfactory. We found that the basic subunits of α-crystallin (B_1 and B_2) had migrated more or less the same distance as the subunits of β-crystallin and as some components of the γ-crystallins. The relative mobilities were in several cases the same within the limits of error and, therefore, we were not able to discriminate between the various subunits of the crystallins.

Applying isoelectric focusing in 6 M-urea containing polyacrylamide gels, we obtained a much sharper distinction of the lens proteins (Fig. 4). In HM-crystallin, polypeptide chains with the same isoelectric points as found for αA_1, αA_2 and αB_2 are visible. A polypeptide corresponding with αB_1 is lacking. Interesting are also the two polypeptides designated as αA_X (band 4) and αB_X (band 10). In our laboratory van Kamp (1973) found two bands with the same isoelectric points as αA_X and αB_X in embryonic bovine α-crystallin. As the patterns of the rabbit and calf α-crystallin polypeptide chains on isoelectric focusing gels are very similar, we assume that αA_X and αB_X from HM-crystallin were originally embryonic α-crystallin polypeptides. Furthermore, polypeptides with the same isoelectric points as some of the β-crystallin chains are obtained (bands 7, 8, 11 and 12, Fig. 4). The occurrence of HM-crystallin is very probably a result of aging processes, implying the formation of aggregates from pre-existing soluble crystallins. From further experiments we know already that HM-crystallin is especially found in large amounts in the nucleus, the older part of the lens. In the case of bovine lenses the same phenomenon was observed by Spector, Freund, Li and Augusteyn (1971a). On SDS polyacrylamide gels, polypeptides with molecular weights ranging from 16 500–39 000 Daltons were found for HM-crystallin (Table II and Fig. 5). Bands corresponding with molecular weights of approximately 16 500 and 17 500 are also found in β_3-crystallin. The heavy band of approximately 21 000 corresponds with the α-crystallin chains. This average molecular weight of the αA and αB chains is in agreement with the findings of Schoenmakers, Gerding and Bloemendal (1969), Spector, Li, Augusteyn, Schneider and Freund (1971b) and Bloemendal, Rotmans-van Poppel and van der Ouderaa (1972) for calf lens subunits. The five bands in the molecular weight range from 23 000–39 000 correspond with polypeptide chains found in the β-crystallins. Furthermore the value found for γ-crystallin is in good agreement with the results of Björk (1964c), obtained with calf γ-crystallin.

Immunoelectrophoretically HM-crystallin revealed two precipitin lines, one in the α- and one in the β-crystallin mobility range [Fig. 2(b)].

These findings lead to the conclusion that HM-crystallin of the rabbit lens is aggregated protein, very likely formed as a result of aging processes and composed of

α-crystallin polypeptide chains with additional β-crystallin chains. Furthermore α- and γ-crystallin of rabbit lens are similar to that from calf lens, whereas certain differences exist between the β-crystallins of the two species.

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HM-Crystallin as an Intermediate in the Conversion of Water-soluble into Water-insoluble Rabbit Lens Proteins

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Gel chromatography on agarose of extracts from cortex and nucleus of the rabbit lens revealed considerable differences in the amounts of α and high molecular weight crystallin. Alpha crystallin, high molecular weight crystallin and urea soluble fraction were compared using sodium dodecyl sulphate gel electrophoresis and isoelectric focusing in 6 M urea. The cortical fractions revealed similar subunit structures. The same holds for the nuclear fractions. However, the subunit structures of the cortex and nucleus fractions differed greatly. On isoelectric focusing nuclear α-crystallin showed 12–13, cortical α crystallin 4 polypeptide chains. Applying sodium dodecyl sulphate gel electrophoresis in the presence of dithiothreitol, cortical α-crystallin revealed two bands, nuclear α crystallin three main bands and four minor bands. Two of the latter bands correspond with those of β crystallins. In urea soluble fractions of cortex and nucleus three additional bands corresponding with polypeptide chains with molecular weights between 52 000 and 65 000 Daltons were obtained. Cortical urea insoluble fraction showed one single band with a molecular weight of 31 000 Daltons, whereas the nuclear urea insoluble fraction revealed four additional minor bands with molecular weights ranging from 19 000–60 000 Daltons.

Possible relationships between γ crystallin, high molecular weight crystallin and urea soluble fraction with respect to the insolubilization of lens proteins are discussed.

1. Introduction

The decreasing amount of α-crystallin and the increasing amount of albumoid found in concentric layers as one approaches the centre of the lens led to the suggestion that α-crystallin is being converted into albumoid in vivo (Krause, 1934).

Evidence has accumulated indicating that the water-insoluble protein (albumoid) of the bovine lens is closely similar to, and derived from, α-crystallin. Several properties, including electrophoretic mobility after solubilization, peptide maps, proteolytic activity, immunological reactions and amino acid composition have been compared (Sirchis, Fromageot and Bernard 1956, Thomann, 1962, Rao, Mehta and Cooper, 1965, Ruttenberg, 1965, Waley, 1965). Recently, however, Manski, Behrens and Martinez (1968) and Mehta and Maisel (1968) suggested that, although α crystallin is the major component of the water-insoluble protein, small amounts of β- and γ-crystallins are also present. By electrophoretic and immunological studies Mehta and Maisel (1967) observed that the insoluble proteins of human and cynomolgus monkey lens were identical in composition to the corresponding α-crystallins. However, recent studies of the amino acid composition of α-crystallin and albumoid of the human lens indicate that they compare closely though there is sufficient variation to suggest that albumoid is not entirely composed of α-crystallin, but probably contains some β- and γ-crystallin as well (Clark, Zigman and Lerman, 1969). With respect to the rat and dogfish lens it has been reported that the insoluble protein is similar to γ-crystallin (Zigman and Lerman, 1968, Lerman, Zigman and Forbes, 1968).

In this study possible relations were searched for with respect to the insolubilization of the soluble proteins into albumoid by comparison of the polypeptide chain composition of α-crystallin, HM-crystallin [high-molecular weight crystallin, an aggregated protein, composed mainly of α-crystallin with additional β-crystallin chains

(Liem-The and Hoenders, 1974)] and albumoid of the rabbit lens. The total water-insoluble protein will be referred to as albumoid, whereas the urea-soluble, reduced protein will be referred to as US and the urea-insoluble membrane fraction will be referred to as UI in this paper.

Furthermore, differences in composition between the cortical and nuclear proteins have been studied. The corresponding extracts were submitted to gel chromatography on agarose, polyacrylamide gel electrophoresis in the presence of SDS and isoelectric focusing.

2. Materials and Methods

Preparation of the water-soluble and water-insoluble fractions

Lenses of 5-month-old New Zealand rabbits were used. After discarding the capsule and vitreous material each lens was weighed. Four whole lenses (approximately 1·5 g total) were stirred slowly in a beaker by means of a plastic-coated magnetic stirring bar in 3 ml of saline–phosphate buffer (0·15 M-NaCl/0·02 M-NaH_2PO_4) pH 7·3 at 4°C for 10 min. To avoid the formation of foam, stirring was done as gently as possible. After this time, the easily removed cortical portion (40% of the total wet weight of the lens) was suspended in the liquid phase and decanted off. The middle layer comprising 40% of the total wet weight was discarded. The heavy, tough nuclei (20% of the wet weight) were homogenized in 3 ml saline–phosphate buffer. The water-insoluble protein was spinned down at 10 000 rev/min (7850 × **g**) at 4°C for 15 min in a Sorvall centrifuge with a SM24 rotor, and then re-suspended and re-centrifuged 10 times, or until the supernatant showed no u.v. absorption at 280 nm. The resultant residue was suspended (5 mg/20 ml) in neutral aqueous 6 M-urea solution containing 0·04% DTT (Dithiothreitol) overnight at 4°C. The remaining insoluble material was centrifuged in a SS34 rotor at 20 000 rev/min (34 000 × **g**) for 20 min at 4°C. The remaining pellet was then re-suspended and re-centrifuged until the supernatant showed no ultraviolet absorption at 280 nm. The urea-insoluble material was finally washed several times with water, and freeze-dried. The urea-solubilized material was re-precipitated by dialysis against four changes of water for 3 days at 4°C and the entire sac content was freeze-dried.

Column chromatography

Gel filtration of the water-soluble protein was done on a BM combination column 20 (Boehringer). The upper half of the column filled with Biogel A-5 m (exclusion limit 5×10^6 Daltons) was separated by a filter from the lower half filled with Biogel A-0·5 m (exclusion limit $0{\cdot}5 \times 10^6$ Daltons). The total length of the column was 125 cm with an inner diameter of 2 cm. The eluting buffer was saline–phosphate buffer pH 7·3 containing 5–6 drops of pHix™ buffer preservative (Pierce Chem. Co. no. 27207) per litre. It was eluted at a constant flow rate of 0·32 ml/min by using a Cenco peristaltic pump. Portions of 2·5 ml were collected with a LKB 7000 ULTRORAC fraction collector. The extinction of the eluate was recorded with a Uvicord III absorption-meter at 280 and 260 nm. Top fractions were pooled, dialyzed and lyophilyzed.

Isoelectric focusing in the presence of 6 M-*urea*

Isoelectric focusing according to Wrigley (1968) was applied, using tubes of 12·5-cm length. Gels of 7·5% acrylamide were made incorporating 6 M-urea and Ampholine pH 5–8. The ampholyte layer on top of the gel contained 0·04% DTT besides urea and ampholyte. Protein samples were made by dissolving 0·5 mg of dry protein in 100 μl of sample solution, which also contained 0·04% DTT. Fifty μl of this solution was layered on top of the gel. Electrophoresis was at 350 V for 4·5 hr after a pre-run of 1 hr. Staining was done according to the method of Malik and Berrie (1972), which obviates destaining afterwards. To avoid carbamylation all urea solutions were freshly prepared without heating.

Gel electrophoresis in the presence of sodium dodecyl sulphate (SDS)

Gel electrophoresis according to Laemmli (1970) was used. Tubes were first siliconized by immersing them in a 2% solution of dichlorodimethyl silan in chloroform for 10 min. Afterwards the tubes were rinsed to dispose of excess siliconizing solution, and then dried. Gels contained 0·1% SDS. Protein samples contained 1% SDS and 0·04% DTT. The electrophoretic run took 3·0–3·5 hr. Afterwards the gels were stained overnight in a Coomassie brilliant blue solution according to Weber and Osborn (1969). Destaining was done at 37°C with several changes during 2 days. The destaining solution was a mixture of 1000 ml methanol, 375 ml acetic acid and distilled water up to 5 l.

3. Results

Gel chromatography

The elution patterns of the water-soluble proteins of cortex and nucleus are shown in Fig. 1. They reveal 6 peaks, comprising HM-, α, β_1-, β_2-, β_3- and γ-crystallin. Pronounced quantitative differences exist between the extracts of cortex and nucleus. The relative percentage of HM-crystallin in nuclear extract is about 40 times as much as that in cortical extract. On the other hand, reduced amounts of α- and β-crystallins are found in the nucleus compared with the cortex, whereas no considerable quantitative difference is found between cortical and nuclear γ-crystallin.

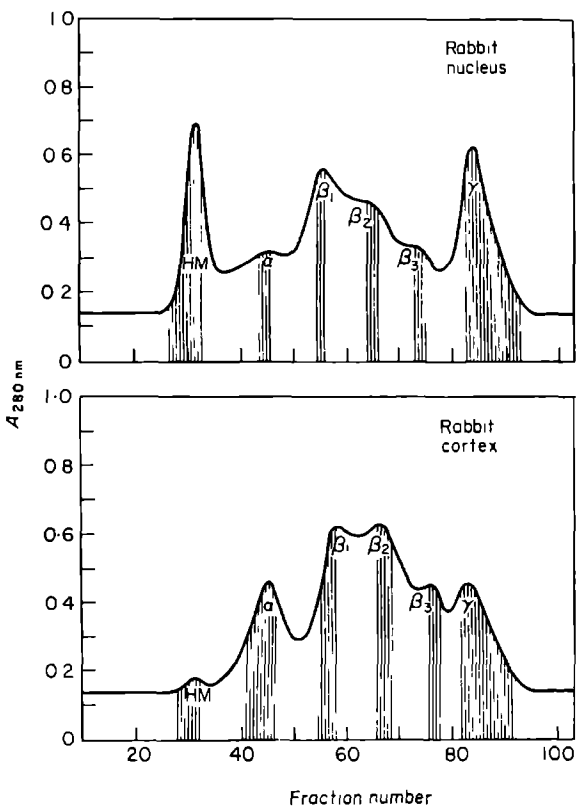

FIG. 1. Column chromatography of lens extracts on Biogel A–5 m/A–0·5 m. The eluted fractions are from left to right: HM-crystallin, α-crystallin, β_1-crystallin, β_2-crystallin, β_3-crystallin and γ-crystallin. Twenty-four mg of protein sample was applied in each fractionation.

Isoelectric focusing in the presence of urea

The gel patterns of isoelectric focusing are presented in Fig. 2. Comparing the α-, HM-crystallin and US fraction of the cortex only slight differences could be detected. The patterns of all three fractions show the four established polypeptide chains of α-crystallin, i.e. αA_1, αA_2, αB_1, αB_2, though in cortical HM-crystallin one clearly visible extra band, i.e. αB_x, is found. Minor amounts of αA_x are seen in the cortical preparations.

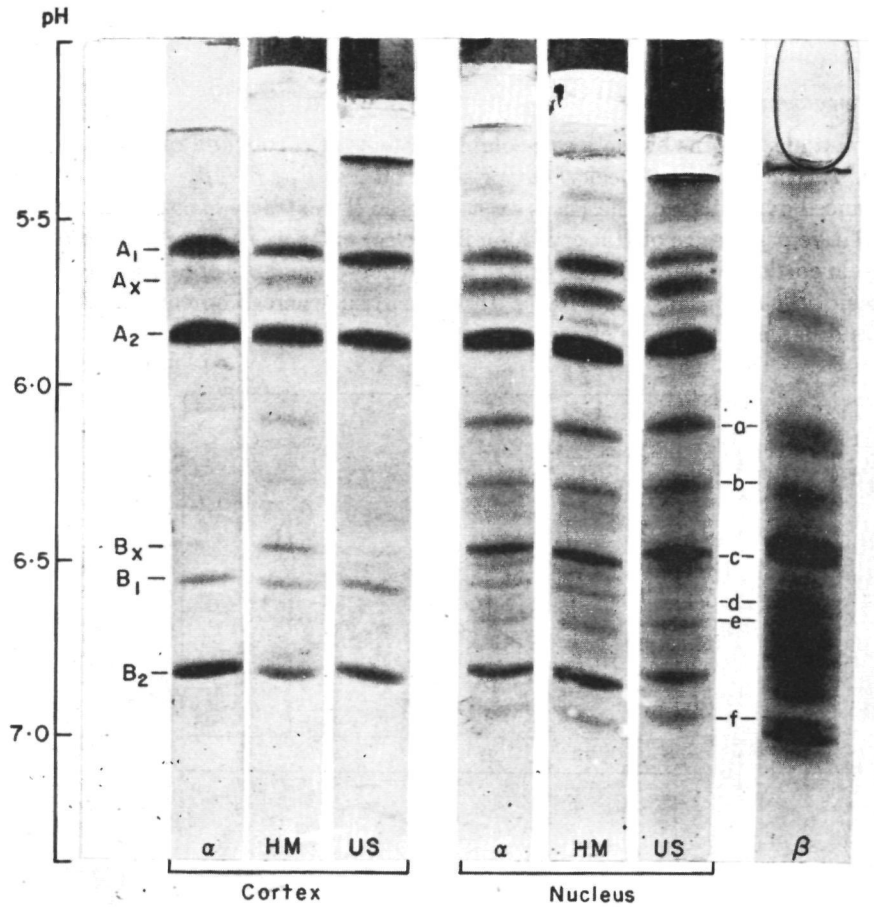

FIG. 2. Isoelectric focusing on polyacrylamide gels in the presence of 6 M-urea and DTT. From left to right gel patterns of cortical α-crystallin, cortical HM-crystallin, cortical US, nuclear α-crystallin, nuclear HM-crystallin, nuclear US and total β-crystallin are shown.
a, b, c, d, e and f are bands corresponding to β-crystallin chains.

The isoelectric patterns of α-, HM-crystallin and US of the nucleus are also almost identical. They reveal 8–9 polypeptides, in addition to the αA and αB chains. Of these polypeptide chains some (a, b, c, d, e, f) have isoelectric points identical to those of some β-crystallin chains. Moreover, we observed αA_x and αB_x in considerable proportions.

No αB_1 was found in HM-crystallin from whole rabbit lens in our previous paper (Liem-The and Hoenders, 1974). The discrepancy is explicable by age differences. In

the foetal and the postnatal rabbit lens, up to 4 months after birth, no B_1 chain can be detected. After 5 months, however, a significant amount of this chain has been formed.

SDS gel electrophoresis

For cortical α-crystallin two polypeptides with molecular weights of 20 000 and of 22 000 Daltons respectively are observed. Cortical HM-crystallin reveals the same

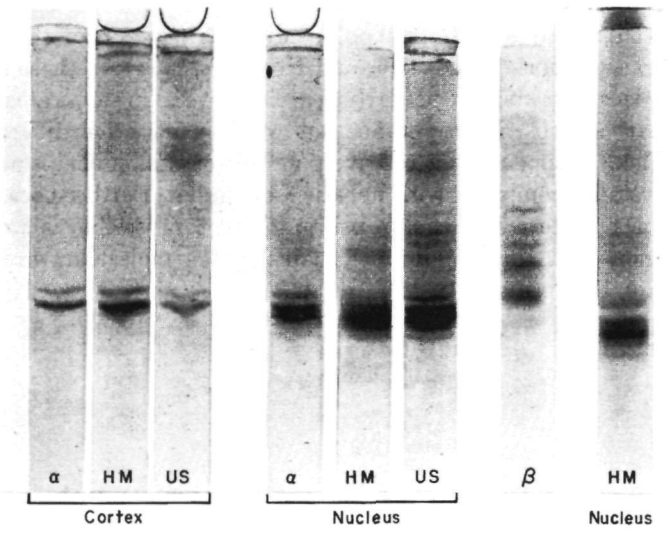

FIG. 3. Sodium dodecyl sulphate gel electrophoresis. From left to right gel patterns of α-crystallin, HM-crystallin and US from the cortex, α-crystallin, HM-crystallin and US from the nucleus, β-crystallin and better resolved nuclear HM-crystallin are shown.

TABLE I

Molecular weights of the polypeptides from α-, β-, HM-crystallin, US and UI

Cortical				Nuclear				
α-Crystallin	HM-Crystallin	US	UI	α-Crystallin	HM-Crystallin	US	UI	β-Crystallin
		65 000				(63 000)		
		60 000				(59 000)	(60 000)	
		54 000			(54 000)	52 000		
						(39 500)		39 000
					(36 000)	35 500		36 000
			31 000	(32 000)	(33 000)	32 000	31 000	32 000
				(29 000)	(29 000)	29 000	(28 500)	28 500
						(25 500)	(25 500)	(26 500)
						(23 000)		23 000
21 500	22 000	22 000		22 000	22 000	22 000		
20 000	20 000	20 000		20 000	20 500	20 500		
				19 000	19 500	19 000	(19 000)	
				(18 000)	(18 000)	18 000		
				(16 500)	(16 500)	(16 500)		

Values of minor components are given in parentheses.

pattern as cortical α-crystallin. However, cortical US shows three additional bands corresponding with molecular weights between 54 000 and 65 000 Daltons (Fig. 3 and Table I). They do not seem to feature in the isoelectric focusing pattern (Fig. 2). It appears that a portion of the sample containing the US fraction did not migrate into the gel, manifesting itself as a dark blue rim on top of the gel.

The nuclear α-crystallin reveals three instead of two main polypeptide chains in the α-crystallin region. Furthermore, four very faint bands are present which can hardly be seen on the photograph. Two of them correspond to polypeptides with molecular weights of 16 500 and 18 000 Daltons, while the remaining two correspond to some of the β-crystallin bands. These additional polypeptide chains were not observed on SDS gel electrophoresis previously (Liem-The and Hoenders, 1974); in those experiments, however, whole lens extracts were used. Nuclear HM-crystallin shows a pattern almost identical to that of nuclear α-crystallin, but with quantitative differences in the β-crystallin region. It appears that relatively more β-crystallin chains are present in nuclear HM-crystallin compared with nuclear α-crystallin. Furthermore, nuclear US reveals a complex pattern with polypeptides in the α-crystallin as well as in the β-crystallin region with three additional chains in the molecular weight range of 52 000 to 63 000 Daltons.

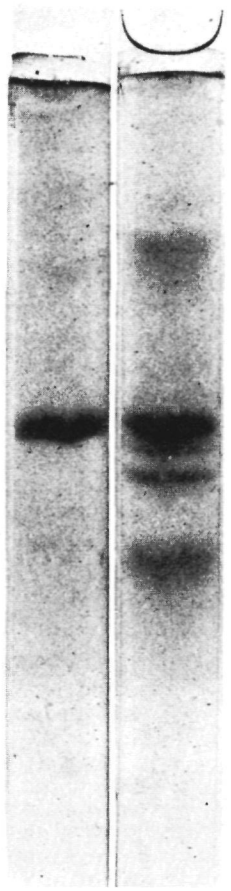

FIG. 4. Sodium dodecyl sulphate gel electrophoresis. On the left the cortical UI and on the right the nuclear UI pattern is shown.

HM-crystallin from whole lens had two subunits with molecular weight of 39 000 and 36 000 Daltons (Liem-The and Hoenders 1974). In the present investigation nuclear HM-crystallin showed only a minor component of 36 000 Daltons, whereas the 39 000 Daltons component is absent (Fig 3 and Table I) In the nuclear US fraction both components, seemingly corresponding to β-crystallin subunits, are now visible In HM-crystallin the 39 000 Daltons band was never found in our last series of experments with 5-month old rabbits At the moment no reasonable explanation for the discrepancy can be given

Finally the solubilized UI fraction showed one single band in the 31 000 Daltons position for the cortical part, whereas four additional minor bands ranging from 19 000 to 60 000 Daltons were found in nuclear UI (Fig 4, Table I).

4. Discussion

Recently, Spector, Li Augusteyn, Schneider and Freund (1971a) and Spector, Freund Li and Augusteyn (1971b) applying molecular sieving techniques found a high-molecular weight protein fraction with molecular weight above 10 000 000 Daltons in calf lens It turned out to be composed of γ-crystallin polypeptides Van Kleef and Hoenders (1973) observed the presence of high molecular weight fractions with sedimentation values between 100 and 190 S and between 30 and 55 S in bovine lenses. These fractions also appeared to be composed of γ-crystallin subunits The present communication shows the similarity in subunit composition of γ-, HM-crystallin and US isolated from cortex respectively from nucleus of the rabbit lens In addition in the nuclear part going from α-crystallin via HM crystallin to the US fraction an increasing amount of β-crystallin chains is involved (Fig 3) As suggested earlier by Spector et al. (1971b), van Kamp (1973) and Stauffer, Rothschild, Wandel and Spector (1974) a transition may take place from α-crystallin to HM-crystallin. In this light, HM-crystallin may be regarded as an intermediate in the process of insolubilization of the crystallins Spector et al (1971b) already proposed that with further aging extensive aggregation or alteration in the chemistry of the lens may occur This would lead to insolubilization and opacification.

Further evidence for the intermediary role of HM-crystallin is obtained by electron microscopic studies Electron micrographs of HM crystallin showed a wide size distribution of aggregated and irregularly shaped protein molecules. Cortical HM-crystallin revealed relatively small aggregates, whereas for nuclear HM-crystallin mainly giant aggregates with dimensions up to 500 nm were found (Liem-The, Stols and Hoenders, in preparation).

It is also interesting to note that in rabbit lenses almost the entire water-insoluble part can be extracted with 6 M-urea in the presence of DTT. The minute residue is the UI fraction, which is apparently membraneous material. UI is completely soluble in 1% SDS +0·04% DTT. On SDS gel electrophoresis cortical UI reveals one band in the 31 000 Daltons position, thus suggesting a simple composition of the fibre membranes with respect to its protein components. Nuclear UI shows, additionally, minor amounts of four other peptide chains, with molecular weights as found in the other nuclear preparations (Fig 4; Table I) Furthermore, differences in polypeptide chain composition between cortical and nuclear proteins were found. It turned out that the polypeptide chain composition of nuclear α-crystallin is more complex than that of cortical α-crystallin On isoelectric focusing nuclear α-crystallin showed additional chains (Fig. 2). Bands a, b, c, d, e and f correspond with chains of the same isoelectric

points as some β-crystallin chains. Another two of them, αA_x and αB_x, are observed in prenatal lenses. They were also seen in α-crystallin from the lens cortex of young animals, up to 4 months old (unpublished results). This suggests that αA_x and αB_x are only present at a young age. With aging they are observed in the inner parts (nucleus) of the lens. In our laboratory a chain with the same isoelectric point as that of αA_x was isolated from calf lens nucleus. It turned out to be an αA_2 chain which is missing 22 amino acid residues at the C-terminus and with a molecular weight of about 18 000 Daltons (van Kleef and Hoenders, in press). Thus, by analogy, we assume that the polypeptide αA_x of rabbit nuclear α-crystallin is a degradation product already formed in early stages. Stauffer et al. (1974) also reported the presence of polypeptides with lower molecular weights than the A- and B-chain in the high molecular weight α-fraction of bovine nucleus. They suggested that limited proteolysis may be involved in the conversion to some of the newly observed chains.

Shapiro (1968) found three bands on SDS electrophoresis of α-crystallin, obtained from whole lens extract of the rabbit. From our study it becomes clear that in rabbit cortical α-crystallin shows two bands, whereas nuclear α-crystallin reveals three main bands and four minor bands on SDS electrophoresis. Consequently, the subunit composition of α-crystallin depends on the location within the lens, as well as on the age of the animal.

On isoelectric focusing apparently the same αB_x chain as found in nuclear α-crystallin is seen in the pattern of cortical HM-crystallin. Since HM-crystallin is obviously formed by aggregation of crystallin molecules, it is probable that cortical HM-crystallin is formed out of earlier synthesized α-crystallin. If a conversion takes place from cortical HM-crystallin into US, then it is rather surprising that the last mentioned fraction does not show this αB_x chain. An explanation might be that in the process of insolubilization newly formed α-crystallin, lacking αB_x, coprecipitates with the giant molecules of cortical HM-crystallin, so as to obscure the αB_x polypeptide, which is present in small amounts.

In the case of nuclear HM-crystallin aggregation seems to incorporate β-crystallin chains in addition to nuclear α-crystallin chains. Here a greater proportion of β-crystallin chains is involved compared to that found in nuclear α-crystallin. For both, the nuclear HM- as well as nuclear α-crystallin the question must be answered whether the β-crystallin polypeptides themselves have aggregated, or whether they formed co-aggregates with α-crystallin.

The observation that US from the cortex (young fibre cells) differs in composition from US from the nucleus (old fibre cells) is in agreement with the findings of Manski and Martinez (1971) who showed immunochemically that the composition of albumoid in bovine lenses is age-dependent. Manski et al. (1968) also showed an increase of some minor β-antigens in the formation of albumoid. The diversity of composition was also observed by Zigman, Schultz and Yulo (1970).

We noted the presence of polypeptide chains between 52 000 and 65 000 Daltons in the US fraction of lens cortex and nucleus by SDS gel electrophoresis (Fig. 3; Table I). Using this method in the presence of DTT it is almost impossible that they are oligomers of the established polypeptides of the rabbit crystallins caused by hydrophobic binding or S-S linkages. One might consider the presence of other stronger covalent linkages between polypeptide chains or that it concerns protein components from cell membranes.

ACKNOWLEDGMENTS

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Chapter 4

Further Characterization of HM-crystallin in Rabbit Lens

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SUMMARY

High-molecular weight (HM-)crystallin was isolated from lens cortex and lens nucleus of the rabbit by column chromatography on agarose. Electron microscope studies revealed the presence of relatively small aggregates in cortical HM-crystallin, obviously oligomers of α-crystallin. On the other hand, nuclear HM-crystallin showed a wide size distribution of large, apparently at random aggregated and irregularly shaped protein molecules. In addition, a considerable proportion of nuclear HM-crystallin, isolated by column chromatography, consisted of particles with a molecular weight above 50 million Daltons. On immunoelectrophoresis it revealed 2 precipitin lines i.e. one in the α-crystallin and one in the β-crystallin mobility range.

On ion-exchange chromatography nuclear HM-crystallin was dissociated into essentially two fractions with sedimentation values of approximately 4 S and 20 S. The first fraction showed characteristics of β-crystallin, while the second fraction was identical to nuclear α-crystallin.

Column chromatography on agarose of the nuclear lens extract of three- to four-month-old rabbits revealed a protein fraction with a sedimentation coefficient of about 18 S, composed of β-crystallin subunits.

Possibilities concerning the architecture of nuclear HM-crystallin are discussed.

1. Introduction

During aging of the bovine eyelens a continuous transition from lower to higher molecular weight protein species appears to take place. Thus, proceeding from the outer surface (young lens fibres) to the centre (old lens fibres), an increasing amount of protein aggregation with a higher molecular weight is found.

Spector, Freund, Li and Augusteyn (1971) have shown that in bovine lenses the number of macromolecules of more than 10 million Daltons increases enormously with age. Further characterization by amino acid analysis and by sodium dodecyl sulphate gel electrophoresis showed no significant differences in subunit composition between high-molecular weight protein and α-crystallin. Studying the crystallins in successive layers of the calf lens, i.e. going from the periphery to the centre of the lens, van Kamp and Hoenders (1973) found increasing amounts of a high molecular weight fraction. Van Kleef and Hoenders (1973) showed the presence of fractions with sedimentation values between 100 S and 190 S and between 30 S and 55 S in calf lenses. In rabbit lenses this high-molecular weight crystallin fraction was also found (Liem-The and Hoenders, 1974a,b). The purpose of this study is to further characterize this high-molecular weight protein.

2. Materials and Methods

Preparation of lens extracts

The method used for isolation of HM-crystallin from the cortex (40% of the lens wet weight) and from nucleus (20% of the lens wet weight) has been described previously (Liem-The and Hoenders, 1974b). Experiments were carried out using lenses of three- to four- and five-month-old animals. Because of the extremely small amounts of HM-crystallin in cortical extracts, some experiments were done only with nuclear HM-crystallin. In the latter case pools of three- to six-month-old lenses were used.

Gel chromatography

HM-crystallin was isolated as described previously (Liem-The and Hoenders, 1974b).

In some experiments fractions with highest concentrations as obtained from the A-5m/A-0.5m column were not lyophilized but concentrated with dry Sephadex G25 coarse. Fractionation of the concentrated preparations was carried out on a column (40 x 2 cm) filled with Biogel A-50m (exclusion limit 50×10^6 Daltons), equilibrated with saline-phosphate buffer (0.15M NaCl/0.02M phosphate) at pH 7.3. The flow rate was 0.13 ml/min using a Cenco peristaltic pump. Fractions of one ml were collected. The effluent was monitored at 280 nm with a LKB Uvicord III absorption meter.

Ion-exchange chromatography

Fractions with highest concentration of nuclear HM-crystallin eluted from the combined Biogel A-5m/A-0.5m column system were pooled and equilibrated against 0.005 M Tris-HCl buffer at pH 8.0 by dialysis. To achieve equilibrium 4-6 changes were needed.

The column filled with DE52 diethylaminoethyl cellulose (Whatman, cat No. 6901) was equilibrated with 0.005 M Tris-HCl buffer at pH 8.0. Elution was performed using a linear gradient from 0.005 M to 0.4 M Tris-HCl. The gradient was formed using a 11300 Ultrograd gradient mixer from LKB. The protein fractions were eluted with a constant flow rate (0.32 ml/min) using a Cenco peristaltic pump. Portions of 2.5 ml were collected with a LKB 7000 Ultrorac fraction collector. The eluate was measured with a Uvicord III absorption meter and recorded.

Electron microscopy

Electron microscope studies were performed using fractions of HM-crystallin from the cortex and the nucleus. Samples were spread on grids coated with carbon film and negatively stained with 0.5% uranyl oxalate (Mellema, van Bruggen and Gruber, 1967) at pH 7.3. Electron micrographs were taken with the Philips EM300 operating at 60 kV at electron optical magnifications of 7 300x and 57 000x. A specimen chamber cooling device has routinely been used.

Sedimentation analysis

Sedimentation coefficients were determined as described before (Liem-The and Hoenders, 1974a).

UV absorption

UV spectra were recorded with a Zeiss RPQ 20 spectrophotometer.

Immunoelectrophoresis

Antibodies to total calf lens protein were isolated from hyperimmunized white New Zealand rabbits. Immunoelectrophoresis was carried out according to the micro-modification of Scheidegger (1955) using LKB apparatus. The sample contained 5% (w/v) of nuclear HM-crystallin. Staining was performed for 15 min in 0.1% Amido Black dissolved in 2% acetic acid. Destaining was done with several changes in 2% acetic acid.

Isoelectric focusing in the presence of 6M-urea

As described previously (Liem-The and Hoenders, 1974b).

Gel electrophoresis in the presence of sodium dodecyl sulphate (SDS)

As described previously (Liem-The and Hoenders, 1974b).

3. Results

Gel chromatography

Column chromatography on Biogel A-50m revealed for nuclear HM-crystallin the elution pattern shown in Fig. 1. The first peak was partially eluted with the void

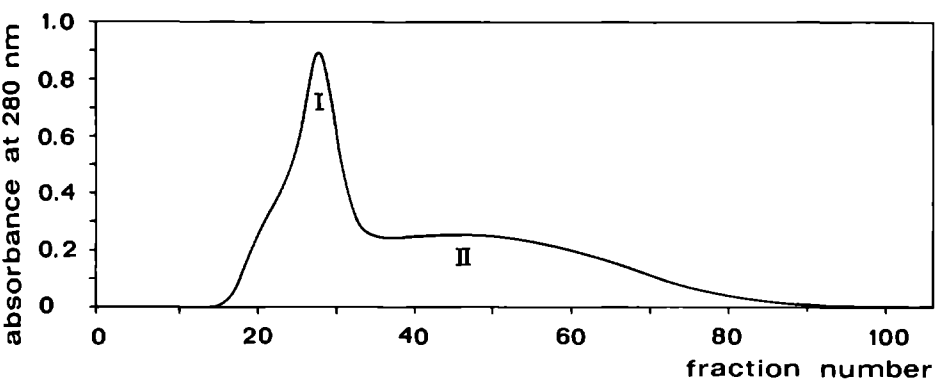

Fig. 1 Column chromatography of nuclear HM-crystallin on Biogel A-50m. Starting material was obtained from 3- to 6-month-old rabbit lenses.

volume, thus indicating that a portion of nuclear HM-crystallin is composed of aggregates of 50 x 10^6 Daltons or larger. It was not possible to measure sedimentation coefficients since the material was too heterogeneous.

Electron microscopy

The electron micrograph of α-crystallin shows molecules of approximately 17nm (Fig. 2). This is in agreement with values obtained for bovine α-crystallin by Bloemen-

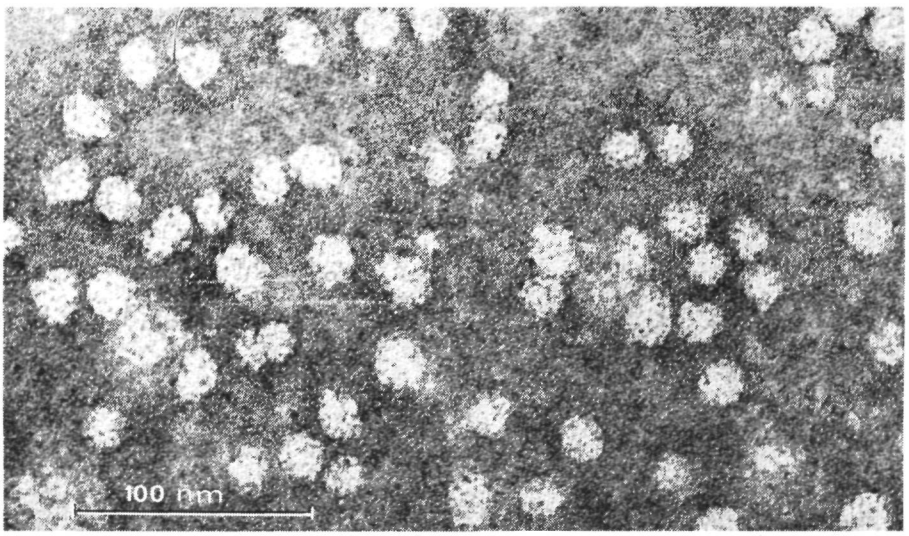

Fig. 2 Electron micrograph of α-crystallin showing spherical molecules with a diameter of approximately 17 nm. Five-month-old rabbits.

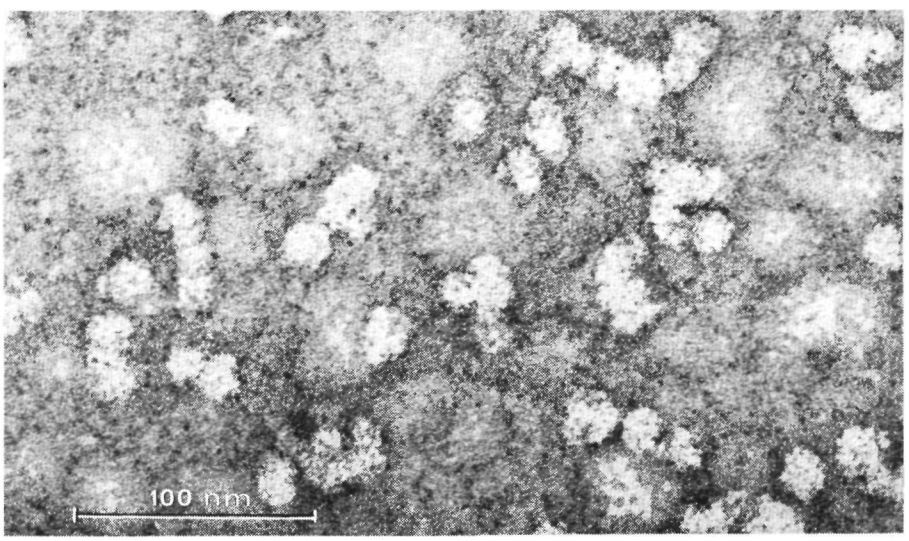

Fig. 3 Electron micrograph of cortical HM-crystallin. It shows mainly small aggregates, obviously consisting of several α-crystallin molecules linked together. Five-month-old rabbits.

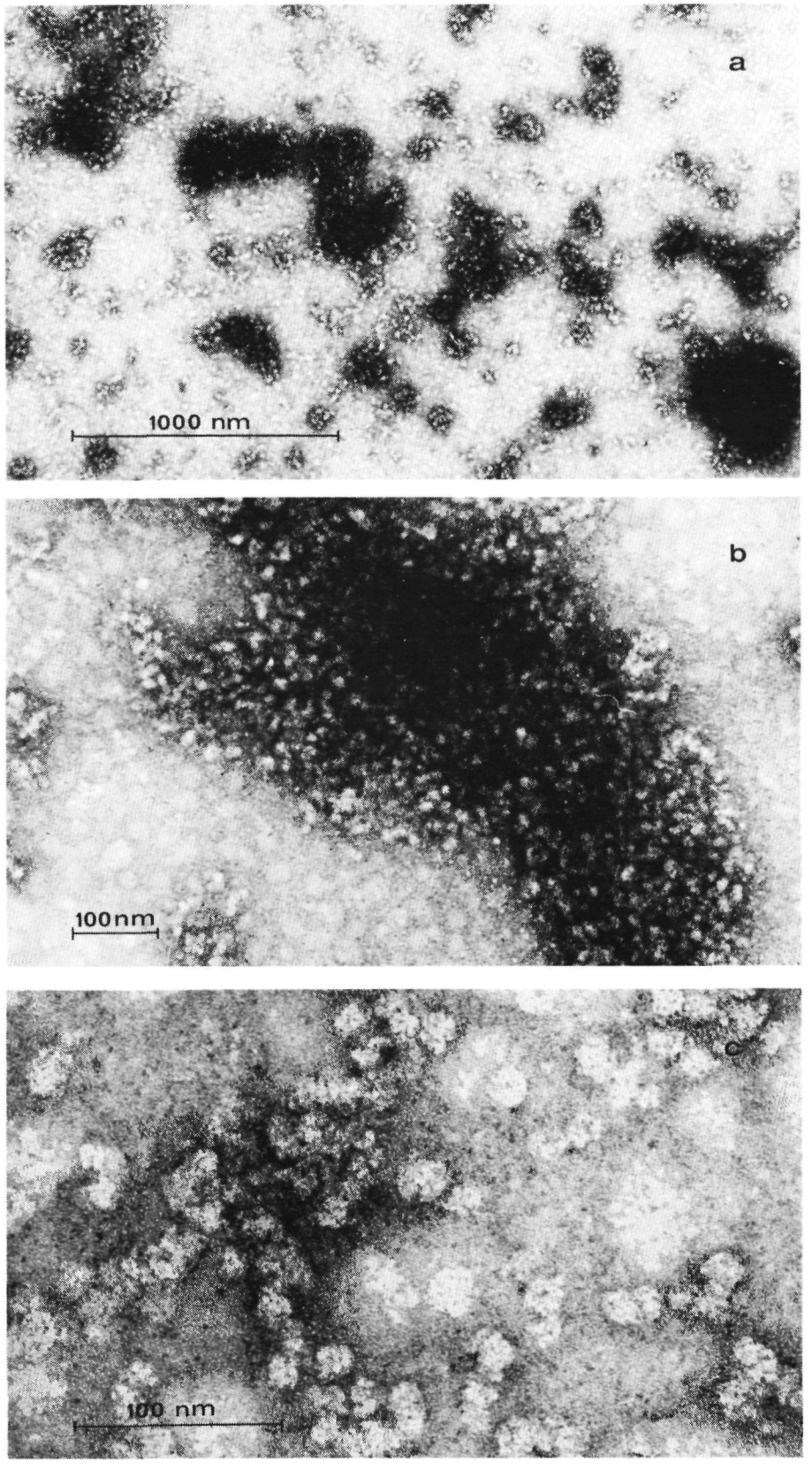

Fig. 4 a, b, c. Electron micrographs of nuclear HM-crystallin at three different magnifications. It shows irregularly shaped, giant aggregates with sizes reaching 500 nm. Five-month-old rabbits.

dal, Berns, Zweers, Hoenders and Benedetti (1972). No difference in size can be found between α-crystallin from cortex and from nucleus. For HM-crystallin, however, aggregates with irregular shape are found. Cortical HM-crystallin reveals mainly small aggregates, obviously consisting of several α-crystallin molecules linked together (Fig. 3). However, in nuclear HM-crystallin aggregates with a great variety of shapes and sizes (up to 500 nm, see Figs. 4a, b and c) are found. This variety suggests that aggregation is a random process. A difference between cortical and nuclear HM-crystallin is that in the former no such giant aggregates were found.

Immunochemical analysis

In Fig. 5 the immunoelectrophoretic pattern of nuclear HM-crystallin is shown. Two precipitin lines were obtained, one in the α-crystallin and one in the β-crystallin mobility range.

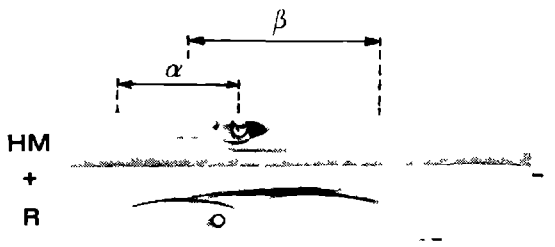

Fig. 5 Immunoelectrophoretic pattern of nuclear HM-crystallin (HM) and rabbit lens extract (R) against anti-calf lens serum. Per hole 2 μl of 5% protein solution was applied. Electrophoresis was performed at 6 V/cm and 4 mA per slide for 3 hr. Five-month-old rabbits.

Ion exchange chromatography

The elution pattern of nuclear HM-crystallin on DE 52-cellulose is shown in Fig. 6. It appears that nuclear HM-crystallin is separated into two couples of incompletely resolved peaks. Single tubes from the ascending and descending side of each couple

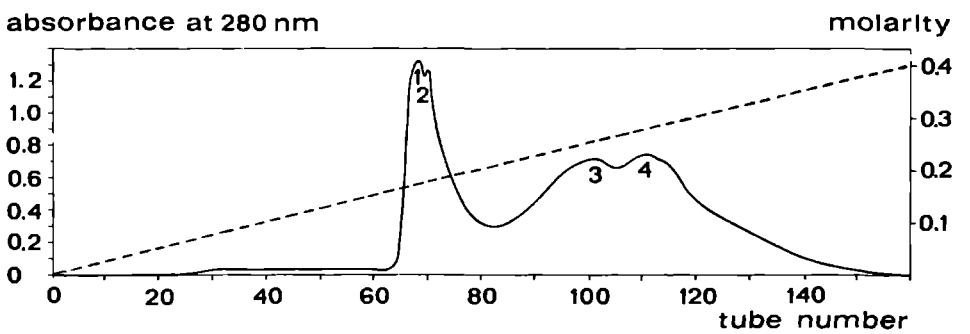

Fig. 6 Elution pattern of nuclear HM-crystallin applying ion-exchange chromatography on DE 52 cellulose. The starting material was obtained from a pool of 3- to 6-month-old rabbits. Recovery was about 85%; additional elution at very high ionic strength and with 0.1 M NaOH revealed some minor components accounting for the remaining 15%. The contents of tubes 66, 70, 99 and 112 were used for sedimentation and spectral analysis.

were submitted to sedimentation and spectral analysis. These samples revealed identical sedimentation coefficients for each pair, i.e. about 4 S (peak 1 and 2) and about 20 S (peak 3 and 4). These values are similar to earlier determined sedimentation coefficients for α- and β_2-crystallin, viz. 18.0 S and 3.7 S (Liem-The and Hoenders, 1974a). Analogous resemblance was shown by the UV spectra (fig. 7). The samples from peak 1

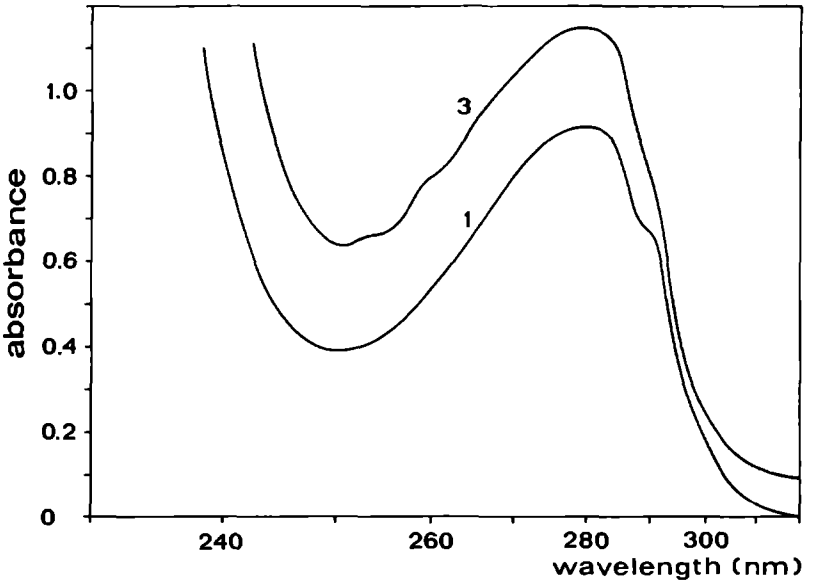

Fig. 7 Ultraviolet spectra of the material in tubes 66 and 99, corresponding to peaks 1 and 3 from the experiment in Fig. 6.

and 2 showed the typical features of β-crystallin, those from peaks 3 and 4 α-crystallin features (compare van Kamp and Hoenders, 1973; for the rabbit crystallins identical spectra are obtained as for bovine lens proteins). In our first experiments isoelectric focusing in 6M-urea was performed using some tubes from the ascending and descending side of each of the pairs of peaks in order to find out the subunit composition. Again identical results were found within each pair. Therefore, in later experiments the material of the poorly resolved peaks was combined. The results obtained with these pools on isoelectric focusing in 6M-urea and SDS gel electrophoresis are shown in Fig. 8. A close resemblance with nuclear α-crystallin was found for (3+4) whereas (1+2) undoubtedly is composed of typical β-crystallin polypeptide chains. The fact that the A_1 chain in nuclear α-crystallin (Fig. 8a) is present in minor amounts seems to be in contradiction with an earlier published pattern (Liem-The and Hoenders, 1974b). However, significant changes in the polypeptide chain composition of rabbit α-crystallin take place at least in the first 5 months after birth (unpublished result). Therefore, the discrepancy has to be attributed to different ages of the lenses used.

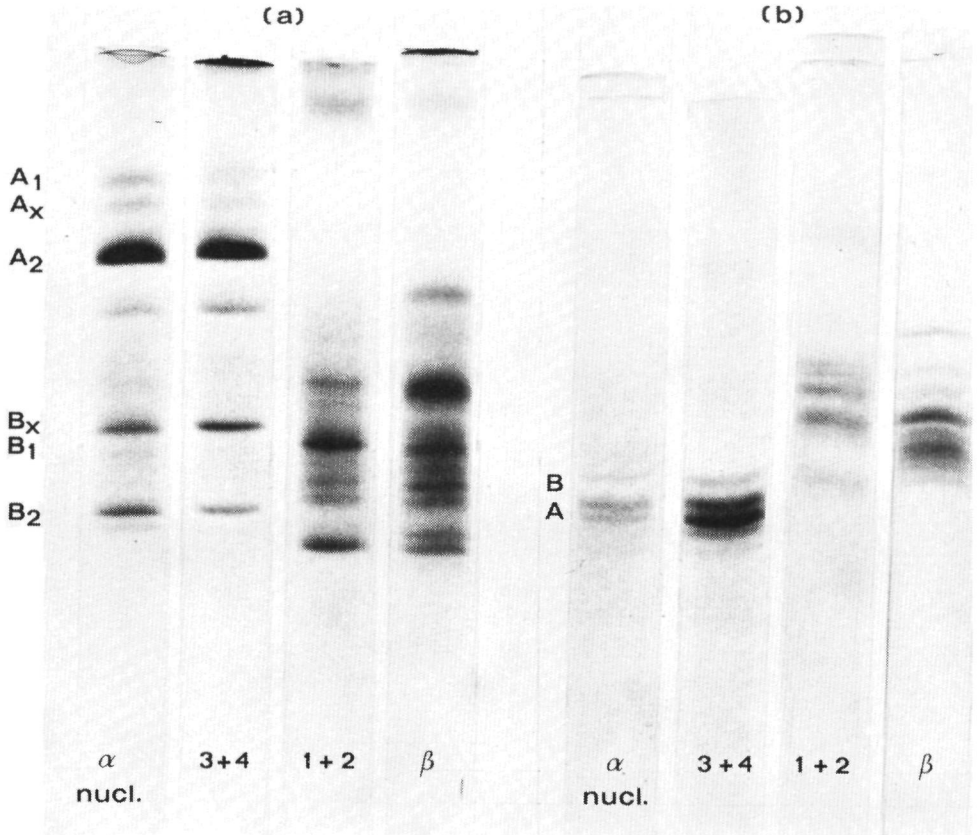

Fig. 8a Isoelectric focusing on polyacrylamide gels in the presence of 6M-urea and 0.04% DTE. From left to right gel patterns of nuclear α-crystallin, combined fractions 3+4 from the experiment in Fig. 6, combined fractions 1+2 from the experiment in Fig. 6, and whole β-crystallin are shown. Preparation of the reference samples according to Liem-The and Hoenders (1974b).

Fig. 8b SDS gel electrophoresis. The same samples as under 8a were applied. The faster bands in $\alpha_{nucl.}$ and (3+4) are presumably degraded polypeptides (Liem-The and Hoenders, 1974b).

To check whether the dissociation of the nuclear HM-crystallin into 4 S- and 20 S-components might be caused by the slightly alkaline medium, a sample was equilibrated with the pH 8.0 buffer during two days. By subsequent chromatography on the Biogel A-5m/0.5m column at pH 8.0 one peak was eluted at the void volume, thus excluding deaggregation of HM-crystallin by the alkaline medium as such.

Experiments with three- to four-month-old rabbits

The elution pattern on agarose Biogel A-5m/0.5m of nuclear lens extract from three- to four-month-old rabbits is shown in Fig. 9. After a medium sized peak for HM-crystallin it reveals two peaks in the position where usually one peak is recorded, namely that of α-crystallin.

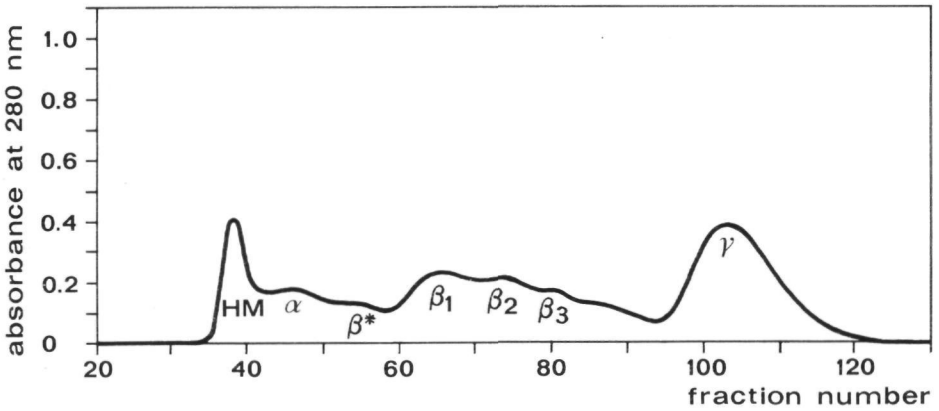

Fig. 9 Elution pattern of nuclear lens extract from three- to four-month-old rabbits obtained by gel chromatography on Biogel A-5m/A-0.5m. From left to right HM-, α-, β*-, β_1-, β_2-, β_3- and γ-crystallin.

Analysis of these two peaks by isoelectric focusing in the presence of 6M-urea and DTE and by SDS gel electrophoresis reveals patterns composed of polypeptide chains of α- and β-crystallin (Fig. 10a, b). The peak designated as α consists of polypeptide chains as they have been found in α-crystallin of lenses from foetuses (unpublished). The sedimentation coefficients of the α and β* are approximately 21 S and 18 S, respectively.

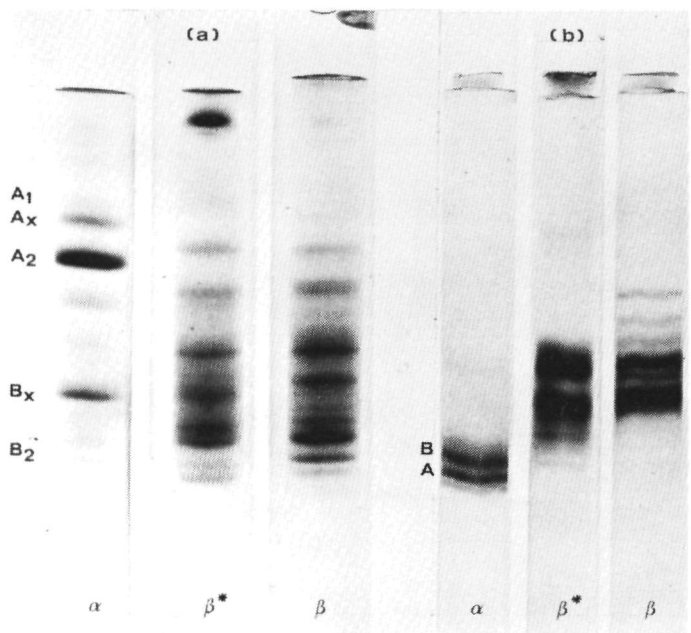

Fig. 10a Isoelectric focusing on polyacrylamide gels in the presence of 6M-urea and 0.04% DTE. From left to right gel patterns of α-, β*- and β-crystallin as obtained from the experiment in Fig. 9 are shown.

Fig. 10b SDS gel electrophoresis. The same samples as under 10a were used.

4. Discussion

Disulfide formation during extraction and fractionation could be responsible for the occurrence of HM-crystallin. Therefore, in a number of experiments 0.04% DTE* was added to the buffer used. Elution profiles identical to those shown earlier (Liem-The and Hoenders, 1974b) were obtained. Thus, it is unlikely that oxidation of sulfhydryl groups interferes with the preparation of HM-crystallin.

By means of electron microscopy we were able to visualize the high molecular weight crystallin particles. It appears that the size of the HM-crystallin particles found in the outer cortex as small, chain-like aggregates (Fig. 3) increases according to the age of the fibre cells. In the nucleus of the lens HM-crystallin occurs in the form of super-aggregates with a wide size distribution, often reaching diameters of approximately 500 nm (Fig. 4). This order of magnitude corresponds with the result obtained by gel chromatography on Biogel A-50m (Fig. 1). The giant particles are irregularly shaped which suggests an at random aggregation of the constituting protein molecules. Furthermore, on immunoelectrophoresis nuclear HM-crystallin revealed two precipitin lines, one in the α- and one in the β-crystallin mobility region.

The question arises how in nuclear HM-crystallin the α- and β-crystallin molecules are arranged. There are several possibilities, among others (Fig. 11):

I) random complexes of single α- and β-crystallin molecules;
II) complexes of α-crystallin molecules and aggregated β-crystallins;
III) a mixture of aggregated α-crystallin and aggregated β-crystallin.

In the light of the result obtained by immunoelectrophoresis one might assume that the arrangement of aggregates in nuclear HM-crystallin is as postulated under III). It should then be possible to separate them on the basis of charge differences. However, HM-crystallin was deaggregated on DEAE-cellulose and fractionated into essentially two components with sedimentation coefficients of 4 S and 20 S, composed of β- and α-crystallin polypeptide chains, respectively. Experiments with lens nuclei of three- to four-month-old rabbits revealed the presence of β-crystallin aggregates with a sedimentation coefficient of 18 S. This finding shows that the β-crystallins are able to form larger aggregates than hitherto supposed. Herewith possibilities II) and III) are more probable than possibility I).

Spector, Stauffer and Sigelman (1973) wondered why human α-crystallin, isolated on a DEAE ion-exchanger, did not give the expected high-molecular weight α-crystallin fraction when fractionated again on Biogel A-15m. However, applying total lens extract directly on Biogel A-15m, the high-molecular weight α-crystallin was observed by these authors. In view of our present results the explanation for this phenomenon might be that the high-molecular weight fraction was deaggregated into lower molecular weight components as a consequence of the ion-exchange step.

* DTE = 1,4-dithioerythritol

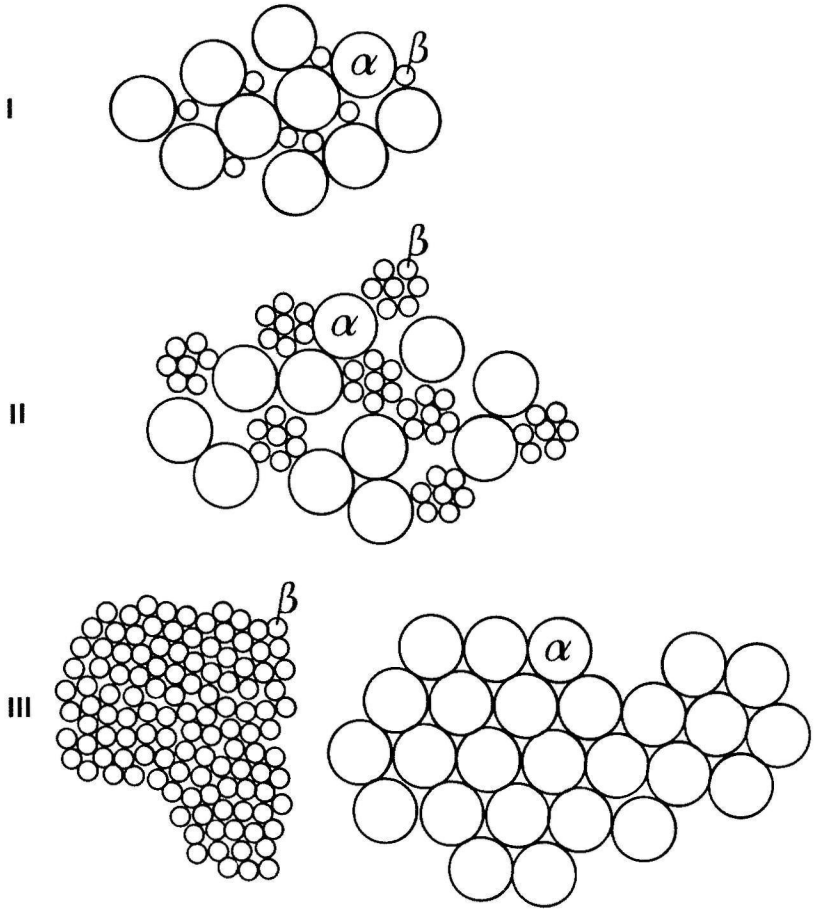

Fig. 11 Schematic drawing representing some possibilities with respect to the arrangement of aggregates in HM-crystallin.

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Chapter 5

X-Ray Induced Cataract in Rabbit Lens

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SUMMARY

Experimental cataracts were obtained by X-irradiation of one eye of two-month-old rabbits with 1800 rad.

In the cataractous lens high-molecular weight (HM-)crystallin is already found in great amounts in the lens cortex, whereas in the unirradiated lens this is true only for the nucleus

In the cataractous cortex and nucleus the subunit structures of α-crystallin, HM-crystallin and US fraction (urea-soluble part of the albumoid) are very complex Their composition are similar to those of the corresponding preparations from the nucleus of the normal lens. They are composed of α- and β-crystallin polypeptide chains. In the case of the US fraction of the cataractous lens also trace amounts of γ-crystallin were observed. On the other hand, relatively simple polypeptide chain compositions were found in the normal lens cortex.

Electron microscopic studies of HM-crystallin from the cataractous lens cortex and nucleus revealed a wide size distribution of irregularly shaped giant aggregates. The same feature was observed for HM-crystallin of the normal nucleus, whereas only relatively small aggregates were seen in HM-crystallin of the normal cortex.

The ultrastructure of the normal lens fibres exhibits an electron dense appearance without any significant demonstrable organelles. On the other hand, the cataractous lens showed pathological changes, as demonstrated by a distinct granular content of the fibres.

Indications for an interpretation of X-ray induced cataract in terms of an accelerated aging process are discussed.

1. Introduction

Cataract due to X-rays has been known for a long time and histological findings have been described in detail for rabbits by Poppe (1942), von Sallmann (1951), Cogan and Donaldson (1951) and many others (for a review, see Lerman, 1964).

Changes of lens metabolism due to X-rays were observed. Pirie, van Heyningen and Boag (1953) found a decrease of reduced glutathione in rabbit lens before any clinical change could be detected. This was confirmed by Hockwin (1962) for bovine lenses. The latter author also observed a decrease in oxygen consumption and he put forward that the course of aerobic glycolysis shows various disturbances.

The most pronounced change seen in mature cataract is the accumulated albumoid; this was observed in X-ray cataract in rabbits by Rupe, Monsul and Koenig (1958) and Orekhovich, Firfarova, Kedrova and Ledikova (1962). Dische and Zil (1951) and Dische (1968) suggested that in rats the formation of disulfide linkages is an important factor during the formation of albumoid, which may take place at the

expense of crystallins or their degradation products: presumably a similar process would occur during the development of irradiation cataract. Orekhovich et al (1962) suggested that the conversion of β- and γ-crystallin to an insoluble state may be due to the oxidation of SH-groups, whereas the cause of the conversion of α-crystallin to an insoluble state is not yet clear.

One may assume that in cataract polypeptide chains are affected. As a result of faulty biosynthesis or metabolic errors changes in the proportions and nature of the polypeptides could occur, which would lead to extensive aggregation and insolubilization. To verify this assumption we have made a comparison of the subunit structures of α-crystallin, HM-crystallin and US fraction from X-irradiated cataractous and control lenses of the rabbit. Experiments were performed using gel chromatography, isoelectric focusing in the presence of urea and sodium dodecyl sulphate gel electrophoresis. Moreover, the water-soluble crystallins were studied by electron microscopy. Finally, the ultrastructural morphology of normal and cataractous rabbit lenses with respect to the damaging effect of X-rays on lens tissue was studied.

In previous papers some characteristics of the crystallins in normal rabbit lenses were communicated (Liem-The and Hoenders, 1974a, b; Liem-The, Stols and Hoenders, 1975). No differences with respect to elution profiles and polypeptide chain patterns obtained from normal and control (unirradiated) lenses were found provided the animals were of the same age.

2. Materials and Methods

Irradiation

New Zealand rabbits were used; all were within a few days of being 8 weeks old at the time of irradiation. The eyes were examined with the slit lamp before irradiation and at various times afterwards in order to determine the course of cataract formation.

The rabbits were immobilized and anesthesized. The area surrounding the one eye to be treated was shielded off by lead plates. Irradiation was carried out with a Philips RT 250. For the experiments, X-rays were delivered with the following factors: potential 250 kV, current 15 mA, half-value layer 3,3 mm Cu, output 70 r/min, focus-target distance 40 cm and field 2 cm in diameter. The exposure time was chosen to give a total dose of 1800 r to the left lens in every case.

The latent period for the onset of cataract was approximately one month. Approximately three months after irradiation the cataracts were mature and both lenses were extracted. The unirradiated lens was completely transparent in all animals used. The lens weights were 500 ± 16 mg and 436 ± 32 mg for control and cataract, respectively.

Isolation of the water-soluble and water-insoluble fractions

The mature cataract is totally opaque and its consistency is pulpy, contrary to the clear control lens, where the cortex is soft and the nucleus tough. For this reason the cataractous lenses could not be divided in cortex and nucleus by means of stirring in a

buffered solution. In order to obtain better defined material for further examination, the whole lens was wrapped in aluminum foil and frozen in a mixture of acetone-dry ice. Forty % of the total wet weight was scraped off and homogenized in 3 ml saline-phosphate buffer (0.15 M NaCl/0.02 M phosphate) at pH 7.3. This part was considered as the cortex. After discarding the middle layer (40% of the total wet weight), the nuclear portion was also homogenized. The water-soluble fraction was separated from the albumoid by centrifuging at 10.000 rev/min (7.850xg) for 15 min at 4°C in a Sorvall centrifuge with a SM 24 rotor.

The water-insoluble protein fraction was then re-suspended and re-centrifuged ten times in saline-phosphate buffer. The resultant residue was suspended in excess of neutral aqueous 6 M-urea solution containing 0.04% DTE (1,4-dithioerythritol) overnight at 4°C. Insoluble material was spinned down in a SS 34 rotor at 20.000 rev/min (34.000xg) for 20 min at 4°C and discarded. The soluble fraction was dialyzed against 4 changes of water for three days at 4°C and the entire sac content was freeze-dried.

The same procedure as above was applied for the normal lenses.

Column chromatography

The water-soluble fractions were isolated as described previously (Liem-The and Hoenders, 1974b). The absorbance at 280 nm of the eluate was measured with a LKB Uvicord III absorption meter and recorded. Fractions with highest concentration were pooled, dialyzed and lyophilized.

Isoelectric focusing

Isoelectric focusing was applied in the presence of 6 M-urea and 0.04% DTE. The method has been described previously (Liem-The and Hoenders, 1974b).

SDS (sodium dodecyl sulphate) gel electrophoresis

As described previously (Liem-The and Hoenders, 1974b).

Electron microscopy

Molecular studies

Electron microscope studies were performed using fractions of α- and HM-crystallin from the control and cataractous lens cortex and nucleus. The method used has been described previously (Liem-The, Stols and Hoenders, 1975).

Morphological studies

For the ultrastructural studies parts of normal and cataractous lenses of rabbits were prefixed in 3% glutaraldehyde in cacodylate buffer pH 7.2, followed by post-fixation in 2% OsO_4 in Veronal-acetate buffer at pH 7.2. Following dehydration in ethanol, the blocks were embedded in Epon 812 (Luft, 1961). Grey sections were obtained with a Reichert Ultramicrotome OM U2 and after double contrasting with uranyl

acetate (Watson, 1958) and lead citrate (Reynolds, 1963) examined in a Philips EM 300.

3. Results

Column Chromatography

The elution patterns of the water-soluble proteins from cortex and nucleus of the normal and cataractous lens are shown in figure 1. In the normal lens six fractions were found namely HM-, α-, β_1-, β_2-, β_3- and γ-crystallin. In the cataractous lens the β-crystallins revealed two poorly separated peaks.

The presence of 0.04% DTE during extraction and fractionation revealed identical results for all lens extracts.

Pronounced quantitative differences exist between the crystallins of normal cortex and nucleus (Table I). As for the cataractous lens, only minor differences could be detected in the elution patterns between cortex and nucleus. Comparison of the normal and cataractous cortices shows drastic changes in the proportions of HM- and β-crystallin. Normal and cataractous nuclei differ in particular in their α- and β-crystallin proportions.

Table I

Distribution of crystallins in normal and cataractous rabbit lens extracts

		HM-crystallin	α-crystallin	β-crystallins	γ-crystallin
lens cortex (outer 40%)	normal	0.57 ± 0.26	16.4 ± 1.3	59.8 ± 3.5	23.2 ± 3.8
	cataract	22.9 ± 4.3	23.9 ± 1.9	33.4 ± 5.0	19.7 ± 0.9
lens nucleus (inner 20%)	normal	20.4 ± 3.1	11.5 ± 0.4	42.4 ± 6.7	25.7 ± 3.6
	cataract	23.9 ± 3.9	21.7 ± 3.3	27.5 ± 4.5	26.8 ± 2.7

Percentages as determined by planimetry of elution patterns (fig. 1) are given. Averaged values of 3 fractionations and their standard deviations are included in the table. No corrections accounting for differences in extinction coefficients of the crystallins were made. The areas measured by planimetry were obtained by drawing perpendiculars in the minima between HM- and α-, between α- and β_1-, and between β_3- and γ-crystallin

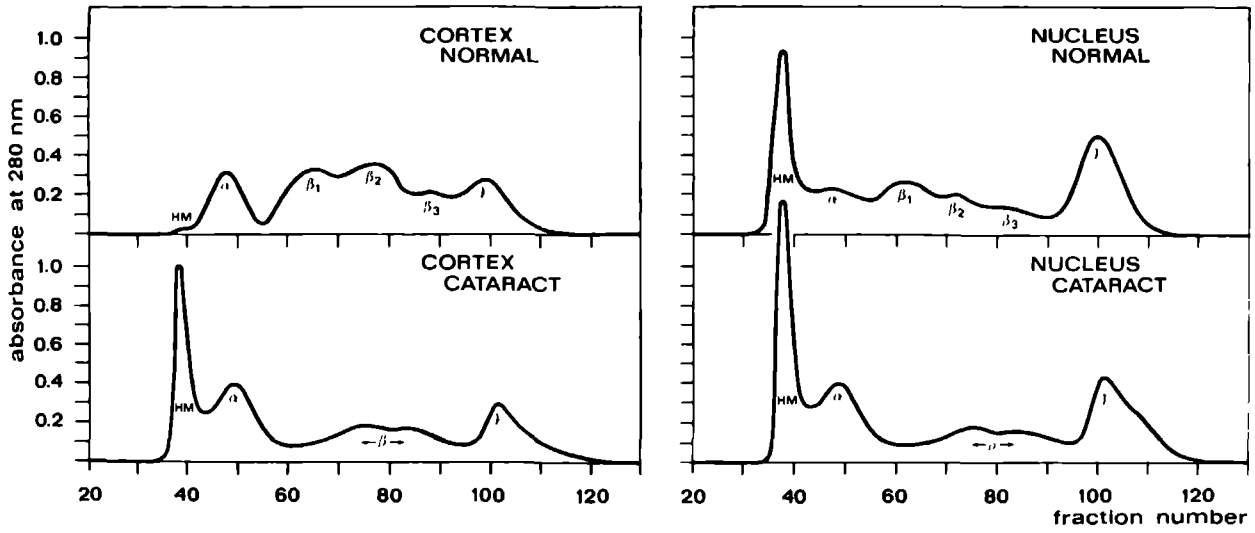

Fig. 1 Column chromatography of the water-soluble fractions from normal and cataractous rabbit lens cortex and nucleus. The eluted fractions are HM-, α-, β_1-, β_2-, β_3- and γ-crystallin. Twenty-four mg of protein sample was applied for each fractionation.

Isoelectric focusing in the presence of urea

The gel patterns obtained by isoelectric focusing are presented in figure 2. Normal cortical α-crystallin, HM-crystallin and US fraction revealed similar, rather simple patterns comprising the four well-known polypeptide chains αA_1, αA_2, αB_1 and αB_2. Especially in cortical HM-crystallin minor amounts of two additional chains, αA_X and αB_X, were seen. Normal nuclear α-crystallin, HM-crystallin and US fraction showed almost identical complex patterns with 8 to 10 polypeptides besides the four cortical α-crystallin chains. Some of these polypeptides (Fig. 2; a, b, c, d, e, f) correspond with β-crystallin chains and, moreover, αA_X and αB_X are present in considerable proportions (Liem-The and Hoenders, 1974b).

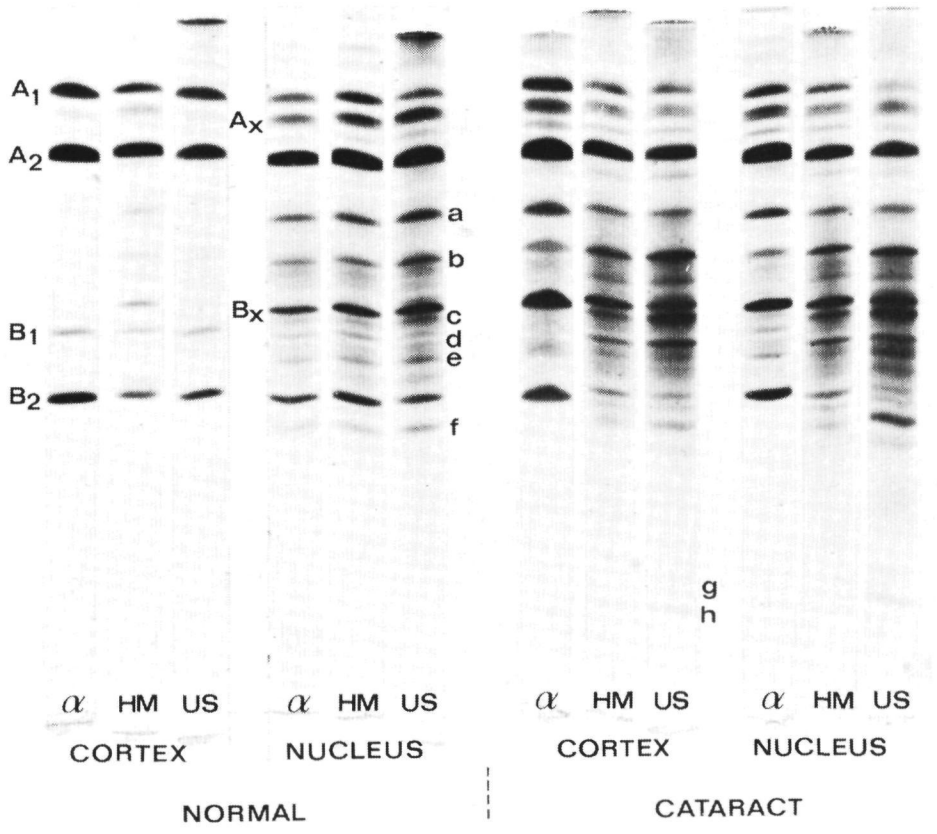

Fig. 2 Isoelectric focusing on polyacrylamide gels in the presence of 6M-urea and 0.04% DTE. From left to right gel patterns of respectively the normal cortical α-, HM-crystallin and US fraction; the normal nuclear α-, HM-crystallin and US fraction; the cataractous cortical α-, HM-crystallin and US fraction, and the cataractous nuclear α-, HM-crystallin and US fraction are shown. Band a-f correspond with β-crystallin polypeptides, g and h with γ-crystallins.

Some of the gels show material left at the origin. In particular the US fraction from normal cortex and nucleus are striking in this respect (see also Liem-The and Hoenders, 1974 b). Comparison with Fig. 3 suggests that the non-penetrating protein may be identical with the high-molecular weight components (ca. 60 000 Daltons) of the SDS-gels.

Only slight differences were observed between the corresponding preparations of cataractous cortex and nucleus. The upper halves of these gel patterns are virtually identical with those of the three normal nuclear preparations. In the lower halves clear differences between the cataractous α-crystallins on the one hand and the cataractous HM-crystallins and US fractions on the other are visible. In comparison with the normal nucleus considerable quantitative, but also qualitative differences are observed with regard to cataractous HM-crystallin and US fraction, although they have many features in common. In cataractous US fraction from cortex and nucleus trace amounts of γ-crystallins are found (bands g and h in Fig. 2).

SDS gel electrophoresis

The gel patterns of SDS gel electrophoresis are shown in Figure 3. The protein fractions obtained from the control lenses revealed patterns identical with those from normal lenses of the same age (Liem-The and Hoenders, 1974b).

The cataractous cortex and nucleus revealed essentially the same gel patterns as normal nucleus for the corresponding preparations. In cataract the proportion of β-crystallin polypeptides seem to be higher, whereas almost no bands in the 50 000 - 65 000 Dalton range can be detected.

Electron microscopy

Molecular studies

Electron micrographs of α-crystallin of normal and cataractous lens cortex and nucleus revealed no size difference (Fig. 4; Liem-The, Stols and Hoenders, 1975). They showed globular molecules of approximately 17 nm in diameter. In HM-crystallin, however, aggregates with irregular shape are present. Similar structures were observed in the micrographs of HM-crystallin of normal lens nucleus and of cataractous lens cortex and nucleus as well. They showed giant particles of different sizes which are apparently aggregated at random. They occur in sizes reaching 500 to 1000 nm (Fig. 5). A different structure was found for HM-crystallin of normal lens cortex. It showed mainly small aggregates consisting of several α-crystallin molecules linked together (Liem-The, Stols and Hoenders, 1975).

Morphological studies

Comparable cortical areas of control and pathologically changed material have been studied.

Cross sections of normal cortex lens fibres demonstrate a more or less regular hexagonal pattern (Fig. 6). Depending on the location of the lens fibres from subcapsular to juxtanuclear, an increasing number of irregularly outlined fibres combined with smaller and deeper identations of the membranes are present.

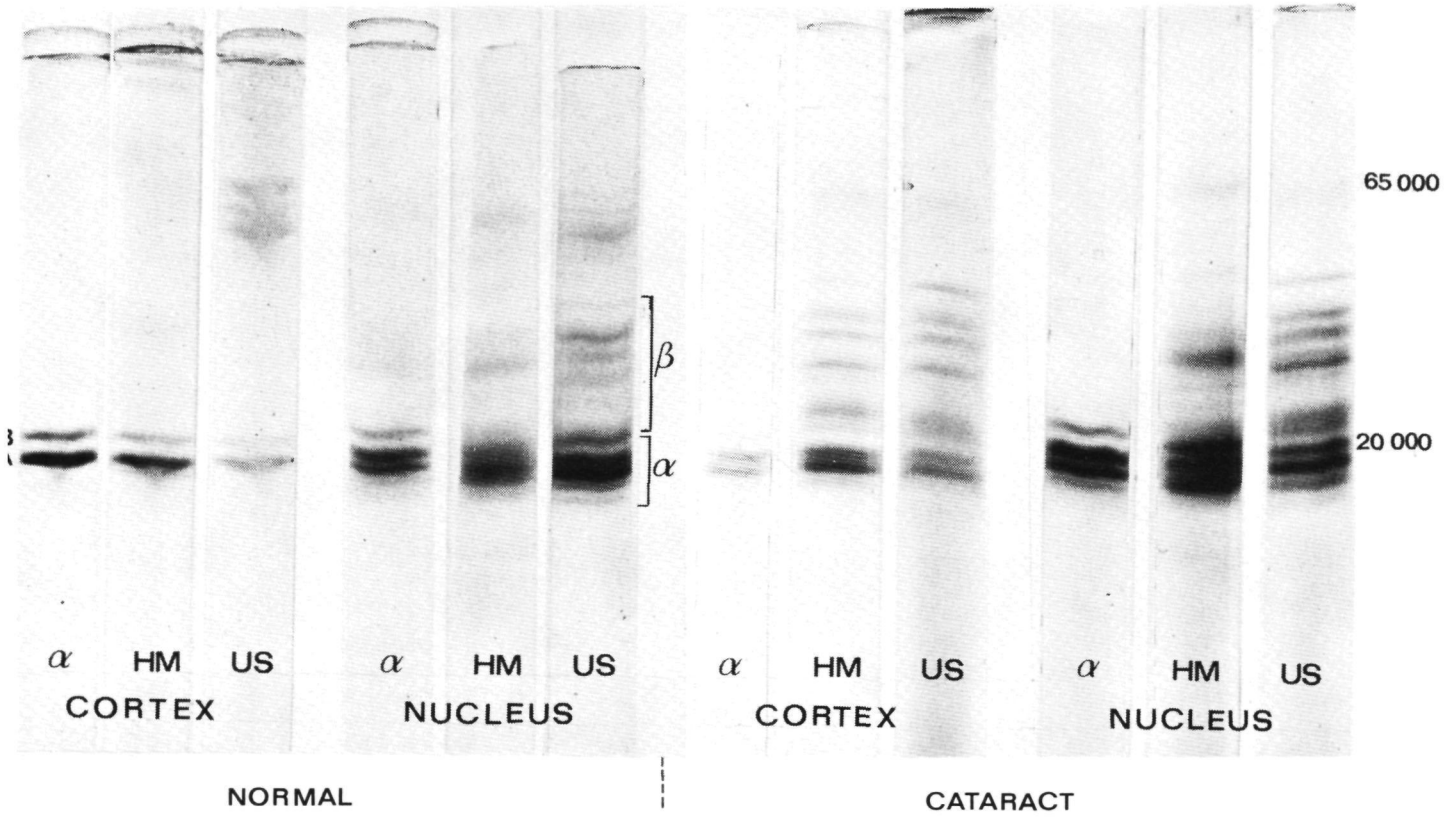

Fig. 3 Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. From left to right gel patterns are shown of respectively the normal cortical α-, HM-crystallin and US fraction; the normal nuclear α-, HM-crystallin and US fraction; the cataractous cortical α-, HM-crystallin and US fraction and the cataractous nuclear α-, HM-crystallin and US fraction.

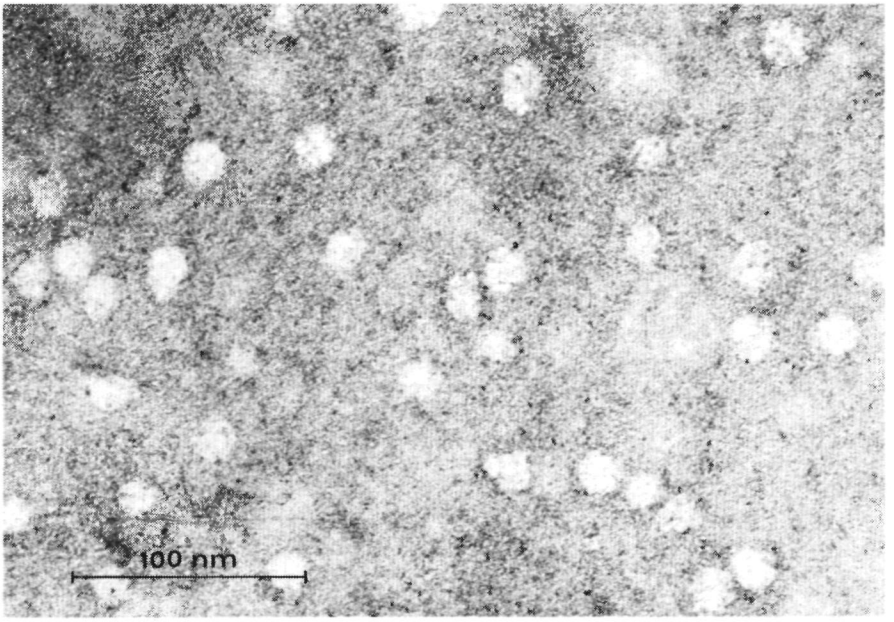

Fig. 4 Electron micrograph of α-crystallin from cataractous cortex.

The normal fibres exhibit an electron dense appearance without any significant demonstrable organelles (Fig. 7). At higher magnifications some small structures can be detected within the greyish, homogeneous, electron dense fibre material. They appear to be scattered ribosomes, cross sections of microtubules and some vesicles. Normal tight junctions are regularly observed.

The experimental cataractous lenses demonstrate distinct pathological changes in areas comparable with the controls. Low magnification micrographs reveal the granular aspect of the cross sectioned fibres (Fig. 8). Differences in electron transparency between the fibres of the control and the cataractous lens are well recognizable.

Compared with the regular pattern of the control, the irradiated fibres appear to be outlined more irregularly. At some places the pattern of the fibres on cross-sections was found to be broken due to the partial loss of the plasma membranes. It results in a long continuous formation of neighbouring fibres (Fig. 9). At higher magnifications the abnormal granularity of the fibre content is evident (Fig. 10). Clusters of interconnected electron dense proteinaceous material fill up the whole fibre. In between, the transparent and empty areas strongly suggest a disintegration of the fibre material. The whole results in a coarse cloudy aspect on the electron micrographs. Various projections demonstrate unrecognizable structures, probably disrupted cell constituents and interdigitations between adjacent fibres. The outlining membranes are mostly intact with well preserved regular distributed tight junctions.

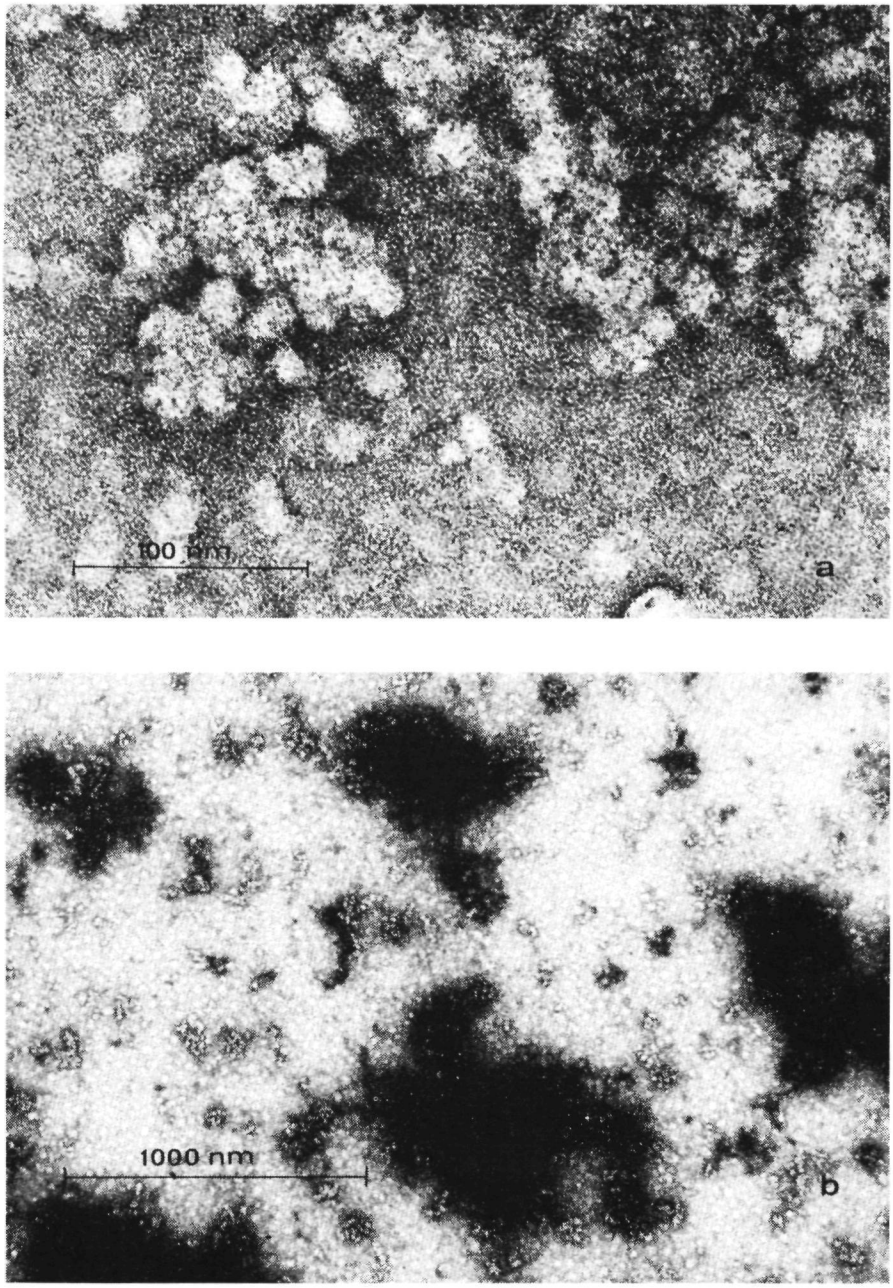

Fig. 5a, b Electron micrographs of HM-crystallin from cataractous cortex at different magnifications.

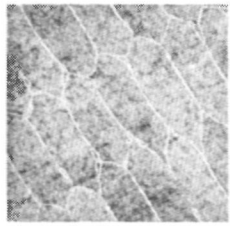

Fig. 6 Cross section of cortex fibres of the control lens. It reveals a more or less regular hexagonal pattern (× 4400).

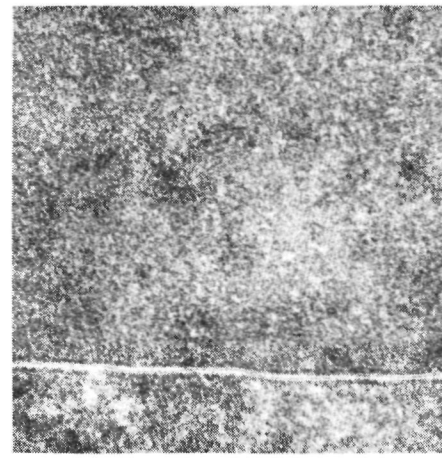

Fig. 7 Detail of two adjacent fibres of the control lens (× 75900).

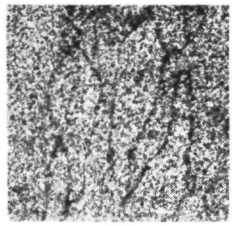

Fig. 8 Cross section of fibres of the cataractous lens, induced by X-irradiation. It shows distinct pathological changes in areas comparable with the controls (× 3500).

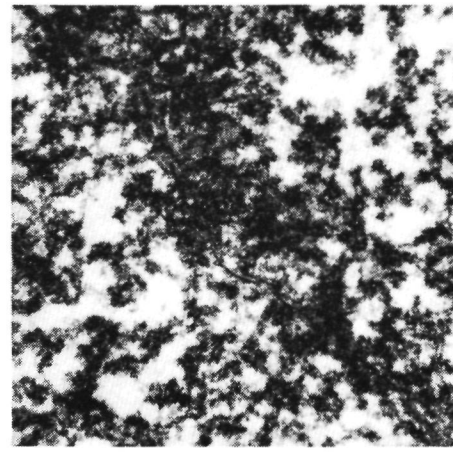

Fig. 10 Detail of two adjacent cataractous fibres The abnormal granularity of the fibre content is evident. Clusters of interconnected electron dense proteinaceous material fill up the whole fibre. In between, the transparent and empty areas strongly suggest a disintegration of the fibre material (× 25000).

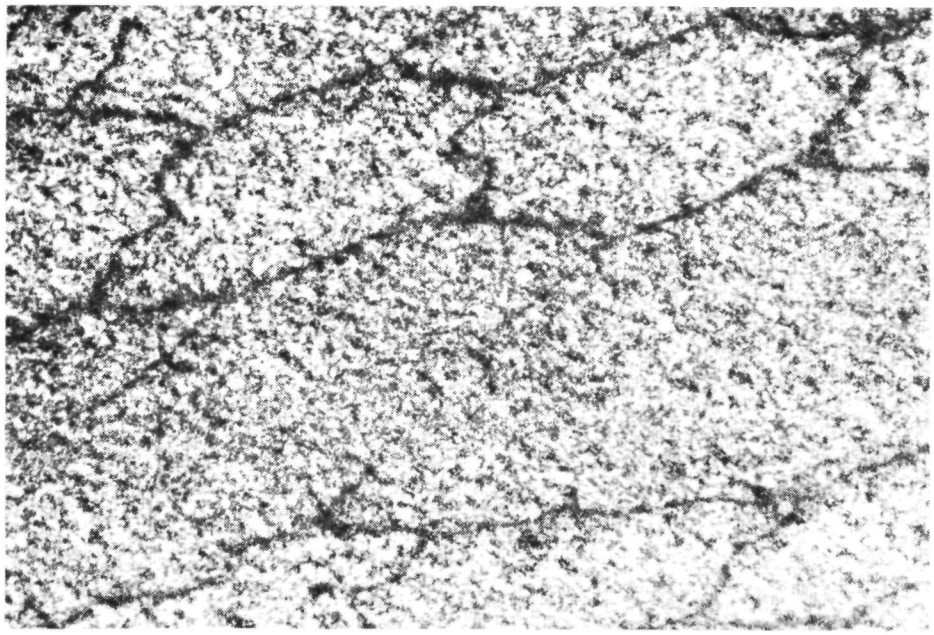

Fig. 9 Cross section of fibres of the cataractous lens. The fibres appear to be outlined more irregularly, compared with the regular pattern of the control lens. At some places the pattern of the fibres was found to be broken due to the loss of the bordering membranes. It results in a long continuous formation of neighbouring fibres (× 7000).

4. Discussion

The distribution of the crystallins in the lens cortex turned out to be drastically changed in X-ray cataract (Fig. 1 and Table I). Only minor differences between the patterns of cataractous cortex and nucleus are visible, whereas a certain resemblance of both patterns with that of the normal nucleus is unmistakable. Most striking is the high content of HM-crystallin in the cataractous cortex and the diminished proportion of β-crystallins in both, cataractous cortex and nucleus (Table I).

The content of US fraction in the whole rabbit lens was found to increase about tenfold in cataract.

These data suggest that in X-ray cataract the process of aggregation and insolubilization occurs at a much faster rate than in normal conditions. In this process HM-crystallin probably acts as an intermediate between the water-soluble proteins and the water-insoluble albumoid (Spector, 1972; Liem-The and Hoenders, 1974b).

Dische (1968) postulated that in case of X-ray induced cataract, inhibition, and eventually cessation of the formation of new fibres of young animals, puts these lenses in some respects in a situation similar to that of the lens of old animals. Hockwin (1962) observed early changes of lens metabolism after X-irradiation, which bear a similarity to the changes produced by increasing age. Orekhovich et al (1962) suggested that radiation cataract resembles an aging process on the basis of their results, showing changes in the SH-group content of soluble lens proteins.

In the present experiments outer cortex (young fibre cells) and nucleus (old fibre cells) of the lens are investigated. Similarities in the polypeptide chain composition of

cortical cataractous α-crystallin, HM-crystallin and US fraction and that of the corresponding preparations from normal lens nucleus are obvious (Fig 2, 3). These and the above mentioned findings of other authors point to the occurrence of accelerated aging in X-ray cataract Of course one should consider the possibility that differences found between nucleus and cortex are not, or not only the result of aging, but also could reflect different proportions of protein synthesized in the lens at different times. For switches in polypeptide synthesis, however, no evidence is available.

In the process of insolubilization α- and β-crystallins are incorporated in the albumoid fraction. This was already postulated in 1949 by Nordmann in his studies on the sclerosis of the crystalline lens. Manski, Behrens and Martinez (1968) found γ-crystallin in addition to α- and β-crystallin in albumoid of normal bovine lens by means of immunochemical methods, whereas in our experiments with rabbit lens the incorporation of γ-crystallin in albumoid was only found in the cataractous lens Similar observations, showing the presence of α-, β- and γ-crystallin in albumoid of human senile cataract were made by York et al.(1972) by immunochemical methods. This also corresponds with the findings of Zigman (1969) and Clark, Zigman and Lerman (1969) who, by amino acid analysis, could not exclude the presence of β- and γ-crystallin polypeptide chains in addition to α-crystallin chains in the albumoid of human senile cataract.

A marked decrease in the concentration of β-crystallins was seen (Table I) Changes in the distribution of the protein component were also observed by Rupeet al (1958) in their studies using moving-boundary electrophoresis On the other hand, Orekhovich et al.(1962), applying preparative zone electrophoresis, did not find significant changes in the proportions of the various protein components in the irradiated lens. The failure to find significant changes was probably due to low X-ray dosages and to the insensitivity of the method used Whether the diminution of the β-crystallins in our cataractous rabbit lens extracts is completely due to insolubilization and incorporation in HM-crystallin, or perhaps partly to leakage through the capsule, is still an open question Current investigations in our laboratory are aimed at the elucidation of this question.

It is peculiar that a significant peak in the α-crystallin position is found in the elution patterns of cataractous lens cortex and nucleus (Fig. 1, Table I). An explanation for this phenomenon may be that β-crystallin during cataract formation is incorporated into HM-crystallin and the US fraction in a rather high proportion (Fig. 3), thus causing a relative increase of the α-crystallin concentration in the soluble protein fraction of the cataractous lens

On the basis of immunochemical evidence, York, Martinez and Manski (1972) concluded that no synthesis of new pathological lens proteins occurs in cataract formation. In view of the multiplicity of only electrophoretically defined polypeptides it is impossible to confirm or to reject this immunochemical conclusion with respect to our data. Considering, however, recent findings in our laboratory (F.S.M. van Kleef, M.J.C.M Nijzink-Maas and H.J. Hoenders, 1974), we tend to the assumption that a very limited number of *de novo* synthesized polypeptide chains is subject to post-synthetic degradation and deamidation steps, thus revealing a great number of derivatives. It is tempting to speculate that in (X-ray) cataract such changes cause aggregation and insolubilization.

The progressive incorporation of chiefly β-crystallin and to a much lesser extent

of γ-crystallin, both having a relatively high SH-content, might suggest that the sulfhydryl group plays a role in cataract formation. This would be in agreement with the findings of many others, e.g. of Orekhovich et al. (1962) and, recently, of Harding (1970, 1972), who estimated a difference in SH-content between normal and cataractous human senile lenses. It may be that by the enhanced incorporation of the crystallins with high SH-content the formation of US fraction is favored due to a higher degree of cross-linking by S-S bridges. The possibility that interchain disulphide formation during preparation could alter the proportions and nature of the crystallin fractions isolated, has been considered in the present study. The presence of DTE did neither change elution profiles (Fig. 1) nor subunit patterns (Fig. 2). This fact not only rules out preparative artefacts, but also indicates that *in vivo* oxidation of sulfhydryl groups in X-ray cataract is of no or only minor importance in the case of rabbits.

The essential result of our electron microscopic studies on molecular level is the finding of very high-molecular aggregates without any apparent structural regularities in the HM-crystallin of cataractous cortex (Fig. 5). In the normal cortex, on the other hand, only relatively small aggregates are visible (Liem-The, Stols and Hoenders, 1975). The impression one obtains from the electron micrographs of HM-crystallin from the normal nucleus as well as from cataractous nucleus and cortex is that of at random clustered spheres of dimensions comparable with that of α-crystallin molecules (Fig. 4). One should, however, keep in mind that the resolving power of the method does not allow to discern β-crystallins.

The ultrastructural studies revealed great differences between normal and cataractous lenses. The granular aspect seen in X-ray induced cataract of rabbit lens is more or less similar to that seen by Philipson (1973) in human nuclear senile cataract. One tends to the assumption that the isolated giant aggregates of HM-crystallin (Fig. 5) are the same as the granular structures observed in the *in vivo* situation (Fig. 10).

We hope to report about the intermediary stages of X-ray induced cataract development in the rabbit lens in a following paper.

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Chapter 6

Changes in the Proportion and Composition of Structural Proteins during the Development of X-ray Cataract in Rabbit Lens

SUMMARY

One eye lens of rabbits was X-irradiated in order to induce cataract. One, two and three months after exposure the irradiated lenses and their controls were studied. Between two and three months after exposure, the total protein content diminished, whereas water-insoluble protein increased. It was found that this loss of protein is reflected by the disappearance of β- and γ-crystallin; a rough estimation revealed a loss of 55% and 65%, respectively.

In regard to the amount of the urea-insoluble fraction no essential change was observed, whereas that of the urea-soluble fraction was found to have increased dramatically.

The water-soluble fraction was fractionated into five to seven fractions by column chromatography. High-molecular weight crystallin content increased progressively with the maturing of cataract. This held true especially for HM-crystallin in the cortex. Moreover, a decrease was observed in the β-crystallin proportion in the late stage of cataract formation.

Age-related, postsynthetic processes appeared to have taken place in the α-crystallin from the control lenses, which was reflected in alterations of its subunit composition. X-irradiation of the lens resulted in an early aging of the cortical α-crystallin, as reflected by the composition of its subunits, whereas nuclear α-crystallin apparently was not affected.

1. Introduction

François and Rabaey (1959), Mach (1963) and Maisel and Goodman (1965) have shown a decrease of the amount of proteins of low electrophoretic mobility in senile cataractous human lenses. By gel chromatography on Sephadex G-100 François, Rabaey and Stockmans (1965) found a preferential decrease of the amount of the low molecular weight proteins. The question arises whether these proteins have leaked out of the lens, or whether they have become insoluble or even were not synthesized anymore. Mach (1963) found a decrease in soluble protein that did not correspond with the increase of insoluble protein in studies on human senile cataract. So, this was indirect evidence for partial loss of protein. Friedenwald (1930) and Gifford, Lebensohn and Puntenny (1932) considered that changes in the permeability of the lens are involved in the process of cataract formation. François and Rabaey (1958) have shown that the isolated bovine lens capsule is permeable only to the lens proteins which migrate slowly in an electric field; some of these are the low molecular size proteins. Charlton and van Heyningen (1968) found a positive correlation between the concentrations of low molecular weight proteins and of inositol in the lens. So, their result does not contradict the idea that low molecular weight proteins are lost from senile cataractous lens by leakage. On the other hand, Sheridan and Zigman (1971), studying the leakage problem in vivo and in vitro, concluded that the conversion of soluble proteins into insoluble forms represents the manner in which soluble protein levels are depressed in the senile cataractous human lenses. Pirie (1968), Clark, Zigman and

Lerman (1969) and Zigman (1969) have shown that a portion of the protein, present in soluble form in normal lenses, is converted to an insoluble form when cataract develops.

Changes in the individual proteins, like an increase in the amount of albumoid and a decrease in the amount of low molecular weight protein, are more pronounced as the cataract matures. It is thought that only the relative proportions and absolute amounts of the proteins are affected in cataract, and not the nature of the individual proteins themselves (Smelser and von Sallman, 1949, Block and Salit, 1946, François, Rabaey, Wieme and Neetens, 1954). This was confirmed by Rupe, Monsul and Koenig (1958), who by investigating the rabbit lens proteins in X-ray cataract by moving boundary electrophoresis, found no evidence of extra components appearing which can account for cataract formation.

More recently, the presence of high-molecular weight protein in the soluble protein fraction was reported by Spector, Li, Augusteyn, Schneider and Freund (1971) in bovine lens. This was confirmed by van Kamp and Hoenders (1973) This high-molecular weight protein was also found in other mammalian lenses (rabbits and humans) by Spector, Stauffer and Sigelman (1973), Maraini and Mangili (1973) and Liem-The and Hoenders (1974a). This aggregated protein is presumed to be an intermediate in the insolubilization process (Spector, Freund, Li and Augusteyn, 1971, and Liem-The and Hoenders, 1974b).

One aim of this study was to establish the degree of aggregation and insolubilization of the soluble crystallins during the development of X-ray cataract The data obtained allowed a calculation about the amounts of crystallins which have disappeared The other aim was the investigation of a possible effect of X-irradiation on the subunit structure of α-crystallin. Three stages were studied, namely one, two and three months after irradiation

2. Materials and Methods

Lenses

The lenses of New Zealand rabbits of approximately two months old were used In addition, the lenses of prenatal animals, 21 days after conception, were studied

Irradiation

The irradiation was performed as previously described (Liem-The, Stols, Jap and Hoenders, 1975b)

Rabbits so irradiated usually showed evidence of lens damage in about 4 weeks, as characterized by the formation of some vacuoles. Approximately three months after irradiation the lenses are totally opaque. The control lens was completely transparent in all stages used. One, two and three months after irradiation four to six animals were sacrificed and the lenses removed.

Determination of soluble and insoluble protein

Four to six lenses of each stage were used.

Each lens was weighed, its diameter and thickness were measured and then it was decapsulated. Afterwards it was lyophilized and its dry weight determined. The water-insoluble part (albumoid) was freed from the water-soluble protein by stirring in saline phosphate buffer at pH 7.3 and centrifuging at 20 000 rev/min (34 000g) using a SS 34 rotor, at 4°C for 20 min in a Sorvall centrifuge. This procedure was repeated until minimal absorption at 280 nm could be measured. Afterwards the water-insoluble protein was washed with demineralized water (3 times) and lyophilized. Its dry weight was determined. The albumoid was separated into urea-soluble (US) and urea-insoluble (UI) fractions. A 6M-urea solution containing 0.04% DTE (1,4-dithioerythritol) was used. The UI fraction was separated from the US fraction by stirring (20 min at 4°C) and centrifuging as above. This procedure was repeated until the absorption at 280 nm was lower than 0.05 for the supernatant. Then the UI fraction was washed with water. After lyophilization its dry weight was determined.

Gel chromatography

Fractionation was performed as described in a previous paper (Liem-The et al, 1975b).

Isoelectric focusing in the presence of urea

This method was performed as described previously (Liem-The and Hoenders, 1974b).

3. Results

Dimensions and weight

The dimensions and weights of the control and exposed lenses are presented in Table I. Similar values were found in the diameters of control and irradiated lenses. However, a gradual increase in thickness corresponding with age was observed in the control lenses. It seems that the same applies to irradiated lenses, with the exception of the mature cataract (three months after irradiation), where a strong decrease was observed. The wet and dry weights of the control lenses increased as a function of age. The same held true for the irradiated lenses between one and two months after irradiation, but between two and three months a drastic decrease of the dry weight was found. Furthermore, a comparison of the dry weight values of the irradiated lenses with those of their controls, especially two and three months after exposure, revealed a decrease.

Table I

Dimensions and weight of control and irradiated rabbit lenses

Lens age (months)	3		4		5	
Time after irradiation (months)	1		2		3	
	control	irradiated	control	irradiated	control	irradiated
diameter (cm)	0.98±0.01	1.01± 0.02	1.02± 0.07	0.98± 0.07	1.04± 0.03	1.04± 0.02
thickness (cm)	0.68±0.02	0.70± 0.02	0.70± 0.01	0.73± 0.07	0.76± 0.04	0.64± 0.03
wet weight (mg)	325±7.6	311±12.4	408±22.7	373±66.7	500±16.1	436±31.7
dry weight (mg)	119±1.2	112± 4.0	162± 6.2	129± 1.8	182± 5.7	93.1 ± 6.6
dry weight (% of wet weight)	36.6	36.0	39.1	35.2	36.4	21.4

Proportion of soluble and insoluble protein

Absolute and relative values of the water-soluble and water-insoluble protein are presented in Table II. A comparison of the absolute values between the control and irradiated lenses, one, two and three months post-irradiation, revealed that the soluble protein content in the exposed lenses were always lower than those of the controls.

In the control lenses an increase was noted in the water-soluble protein content. Furthermore, a marked decrease in the water-soluble protein content concomitant with a drastic increase of the insoluble protein content was observed in the exposed lenses, from two to three months after irradiation; considering the percentages of the dry weight, the increase of the water-insoluble protein was about 40 times, whereas about one half of the water-soluble protein was left.

The increase in water-insoluble protein content was not only found in cataractous lenses, but to a lesser degree also in the five-month-old control lenses; thus, the insoluble protein content in mature cataract, calculated as a percentage of the dry weight, was about tenfold the value of the control.

In Table III the absolute and relative values of the urea-soluble and urea-insoluble proteins are given. It is apparent that the value of the UI fraction is approximately the same in the control as well as in the irradiated lenses. Remarkable here is the increase of US fraction, three months post-irradiation, of control and irradiated lenses.

Gel chromatography

Elution patterns of the water-soluble protein from cortex and nucleus of rabbit lenses of different stages of cataract development and their respective control lenses and an additional pattern of prenatal lenses are shown in Fig. 1.

Table II

Proportions of water-soluble and -insoluble protein of control and irradiated rabbit lens

Lens age (months)	3		4		5	
Time after irradiation (months)	1		2		3	
	control	irradiated	control	irradiated	control	irradiated
Absolute values (mg)						
water-insoluble protein	3.93±028	2.55±0.68	2.66±0.28	2.04±0.22	12.6±1.4	56.7±8.3
water-soluble* protein	115.1	109.5	159.3	127.0	169.4	36.4
% of dry weight						
water-insoluble protein	3.30	2.28	1.64	1.58	6.92	61.0
water-soluble protein	96.7	97.7	98.4	98.4	93.1	39.0

* It was assumed that the total protein content equals the dry weight. These values were obtained by subtraction of the values of WI fraction from those of the dry weight.

In the lens cortices and nuclei of the control as well as the exposed lenses generally six fractions were found, namely HM-, α- β_1- β_2-, β_3- and γ-crystallin.

A major difference between the experimental and control cortices are the amounts of HM-crystallin. In the lens cortex of the mature cataract a great amount of HM-crystallin was observed, whereas no appreciable quantities of it could be found in the other cortical fractions.

The elution patterns of the lens nuclei of the irradiated and control lenses revealed five to seven peaks, namely HM-, α- (β*-, β_1-, β_2-, β_3-) and γ-crystallin. The β* peak was only found in the nuclei of the three-month-old lenses (control and irradiated) and in the four-month-old control lens (Liem-The, Stols and Hoenders, 1975a). However, one β-crystallin peak less was observed in the elution pattern of the lens nucleus in mature cataract.

Furthermore, a gradual increase in the HM-crystallin peak could be observed from the three-month-old to the five-month-old lens.

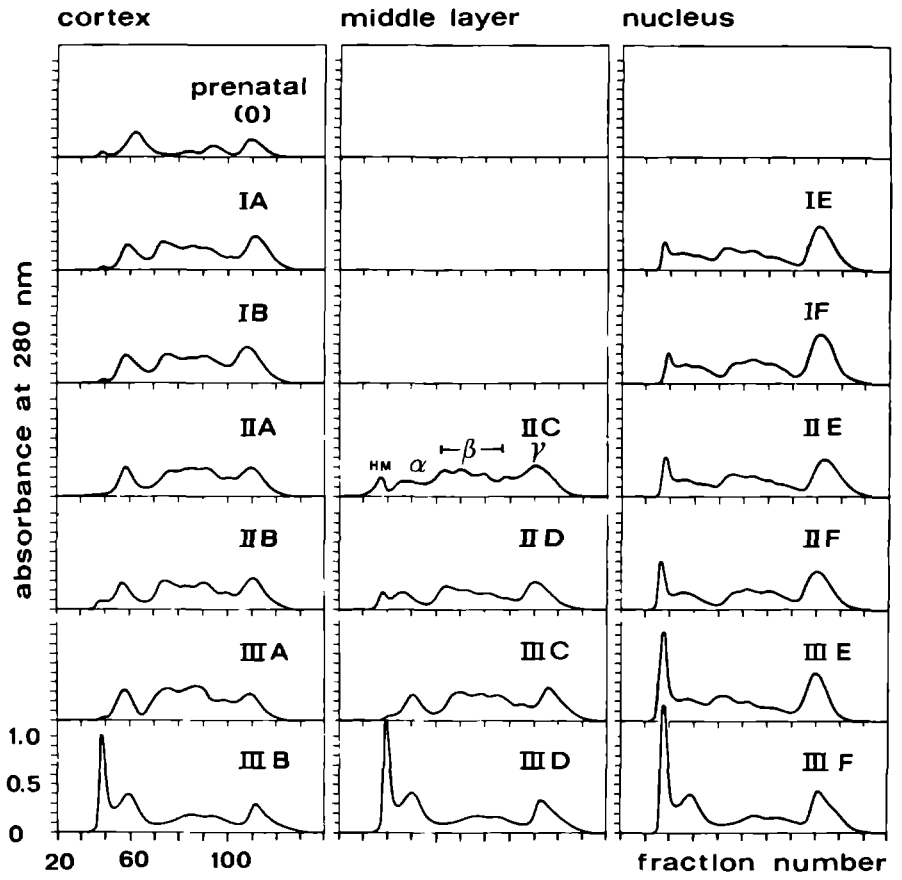

Fig 1 Elution patterns of water soluble lens protein from the cortical, middle (only in some cases) and nuclear parts of control as well as irradiated lenses, fractionated by gel chromatography on Biogel A-5m/A-0.5m. They reveal peaks for HM-, α-, β- and γ-crystallin Elution patterns IA, IIA and IIIA are from the cortical parts of three-, four- and five-month-old control lenses, respectively, IB, IIB and IIIB those from the irradiated lens cortex, one, two and three months after exposure, respectively Diagrams IIC and IIIC are from the middle layers of four- and five-month-old control lenses, respectively, IID and IIID from the exposed lens, two and three months after irradiation, respectively Patterns IE, IIE and IIIE are derived from three-, four- and five-month-old control lens nucleus, whereas IF, IIF and IIIF are those from the irradiated lens nucleus, one, two and three months after exposure, respectively. In addition an elution diagram of prenatal whole lens extract is shown (0)

Table III

Proportions of urea-soluble and -insoluble protein of control and irradiated rabbit lenses

Lens age (months)	3		4		5	
Time after irradiation (months)	1		2		3	
	control	irradiated	control	irradiated	control	irradiated
Absolute values (mg)						
urea-insoluble (UI)	1.49±0.18	1.39±0.29	1.26±0.31	0.94±0.23	2.07±0.45	1.06±0.37
urea-soluble (US)	2.44	1.16	1.40	1.10	10.53	55.64
% of dry weight						
urea-insoluble (UI)	1.26	1.25	0.77	0.73	1.13	1.14
urea-soluble (US)	2.05	1.05	0.87	0.85	5.79	59.69

A comparison of the relative percentages of soluble proteins in normal and irradiated rabbit lens cortex and nucleus are given in Table IV. The values were obtained from planimetric measurements. They were not corrected for differences in extinction coefficients.

Cortical HM-crystallin of the control lenses occurred in extremely small quantities, whereas in the irradiated lenses, two months post-irradiation, the HM-crystallin content was three times as much as that from one month post-irradiation. An even more dramatic increase was observed for cortical HM-crystallin from irradiated lenses two to three months after exposure.

Cortical β-crystallin of the control and exposed lenses showed similar values, except in the mature cataract, where a marked diminution was found. For nuclear β-crystallin the same was found as in cortical β-crystallin.

A comparison of cortical and nuclear γ-crystallin revealed higher values for the latter, which is consistent with the observations in bovine lenses (amongst others by van Kamp and Hoenders, 1973).

Gamma-crystallin was eluted at about the same volume as nucleotides on Biogel A-5m/0.5m. These nucleotides could be separated from γ-crystallin by column chromatography on Sephadex G 200. A correction has been made for the presence of the nucleotides (see legends in Table IV). This correction was not made in our previous papers (Liem-The and Hoenders, 1974a and b;Liem-The et al., 1975b).

Table IV

Percentages of crystallins in cortex and nucleus of control and irradiated rabbit lenses.
The values of γ-crystallin of lens cortex, middle layer and nucleus are corrected in order to account for the presence of nucleotides. Planimetric measurements of the elution patterns, obtained after fractionation by column chromatography on Sephadex G 200, revealed that about 20% are due to nucleotides. No corrections were necessary for the values of γ-crystallin of the middle layer and nucleus, because here the amounts of nucleotides present are negligible.

Lens age (months)		3		4		5	
Time after irradiation (months)		1		2		3	
		control	irradiated	control	irradiated	control	irradiated
Cortex	HM	0.90±0.12	1.06±0.37	1.26±0.46	3.45±0.28	0.60±0.26	24.0±4.3
	α	18.9 ±1.4	20.2 ±0.6	18.1 ±0.1	20.0 ±1.9	17.4 ±1.3	25.1±1.9
	β	59.7 ±2.1	57.2 ±0.6	61.9 ±2.3	59.7 ±1.2	63.4 ±3.5	35.1±5.0
	γ	20.5 ±1.2	21.5 ±0.6	18.7 ±2.4	16.9 ±2.9	18.6 ±3.8	15.8±0.9
Middle layer	HM	-	-	4.91	5.24	0.78	26.3
	α	-	-	15.6	16.4	16.0	24.9
	β	-	-	52.4	48.8	54.3	26.3
	γ	-	-	27.1	29.5	28.9	22.5
Nucleus	HM	7.8 ±0.8	7,6 ±0.7	11.7 ±0.6	14.6 ±1.9	20.4 ±3.1	23.9±3.9
	α	16.2 ±1.9*	17.8 ±0.7*	15.8 ±0.2*	14.5 ±2.3	11.5 ±0.4	21.7±3.3
	β	37.8 ±0.9	35.6 ±1.7	41.3 ±1.6	38.5 ±2.7	42.4 ±6.7	27.5±4.5
	γ	38.2 ±1.0	39.0 ±0.8	32.1 ±2.4	32.9 ±2.5	25.7 ±3.6	26.8±2.7

* These values comprise α and β*

Calculations

Calculations referring to leakage of the various crystallins are presented in Tables V and VI. Some explanations with regard to the calculations in the tables are given here.

First, we have to calculate the contribution of each of the crystallins in the total lens. For this purpose we have to total up the amounts of the crystallins found in the different lens parts (see also Table IV); in addition we must multiply them by 0.4, 0.4 and 0.2, respectively, according to the contribution of the cortex (40%), middle layer (40%) and nucleus (20%). In order to know their real proportions, the figures obtained above were divided by their respective extinction coefficients ($A^{1\%}_{280}$ values of 25, 10, 22 and 28 were determined for HM-, α-, β- and γ-crystallin). The absolute amount of the water-soluble protein is known (Table II) and so the absolute amount of each of the crystallins can be calculated.

Table V

Proportions of lens proteins in the control and irradiated lenses, two and three months after irradiation

Lens age (months)		4		5	
Time after irradiation (months)		2		3	
		control	irradiated	control	irradiated
Total lens (%)	HM	4.80	6.39	4.64	24.90
	α	16.64	17.47	15.67	24.34
	β	53.98	51.08	55.56	30.06
	γ	24.74	25.13	24.14	20.67
Total lens corrected (%)	HM	3.67	4.97	3.60	18.01
	α	32.05	33.46	30.49	43.94
	β	47.30	44.36	49.12	24.70
	γ	17.00	17.21	16.77	13.35
Total crystallins (mg)	HM	5.85	6.31	6.10	6.56
	α	51.06	42.49	51.65	15.99
	β	75.35	56.34	83.21	8.99
	γ	27.08	21.86	28.41	4.86
Total US (mg)	HM	-	-	-	-
	α	0.98	0.77	7.37	36.20
	β	0.42	0.33	3.16	16.70
	γ	-	-	-	2.78
Total lens protein (mg)	HM	5.85	6.31	6.10	6.56
	α	52.04	43.26	59.02	52.19
	β	75.77	56.67	86.37	25.69
	γ	27.08	21.86	28.41	7.64

Table VI

Lens proteins of the exposed lenses, two and three months post-irradiation

Time after irradiation (months)		2	3	Change (%)
Total lens protein (mg)	α	43.26	52.19	+ *
	β	56.67	25.69	− 54.7
	γ	21.86	7.64	− 65.1

* The increase of α-crystallin between two and three months after irradiation was not significant.

Based on scannings of the SDS- (sodium dodecyl sulphate) and isoelectric focusing gel patterns (Liem-The et al., 1975b) an estimation was made of the relative proportion of the crystallin contribution to the US fractions of the control as well as the irradiated lenses. This proportion was 70% for α-crystallin and 30% for β-crystallin in US fraction of the five-month-old control lenses. These percentages also apply to the four-month-old lenses. For the mature cataract these proportions were 65% α-crystallin, 30% β-crystallin and 5% γ-crystallin. The absolute amount of total US is known (Table III), thus the asbolute amounts of the crystallins can be calculated.

HM-crystallin was not included in the calculations of loss of crystallins, because of its approximately equal amounts in control as well as in irradiated lenses. On account of assumptions made, the values found are only rough estimations.

Isoelectric focusing in the presence of urea

The isoelectric focusing gel patterns of the α-crystallins of the whole prenatal lens and of the cortices of control and exposed lenses are presented in Fig. 2. In the prenatal α-crystallin two polypeptide chains, namely αA_2, and in minor amounts αB_X, were observed (Liem-The and Hoenders, 1974a).

Alpha-crystallin from the three-month-old control lenses revealed a pattern with three bands, corresponding with αA_X, αA_2 and αB_X. In four-month-old control lenses five subunits, i.e. the three just mentioned ones and αA_1 and αB_2, were found. Alpha-crystallin from five-month-old control lenses showed four subunits, αA_1, αA_2, αB_1 and αB_2; whereas αA_X and αB_X have disappeared.

In the one month post-irradiated lens α-crystallin revealed a pattern with four bands, corresponding with αA_1, αA_X, αA_2 and αB_X. Similar patterns were found for

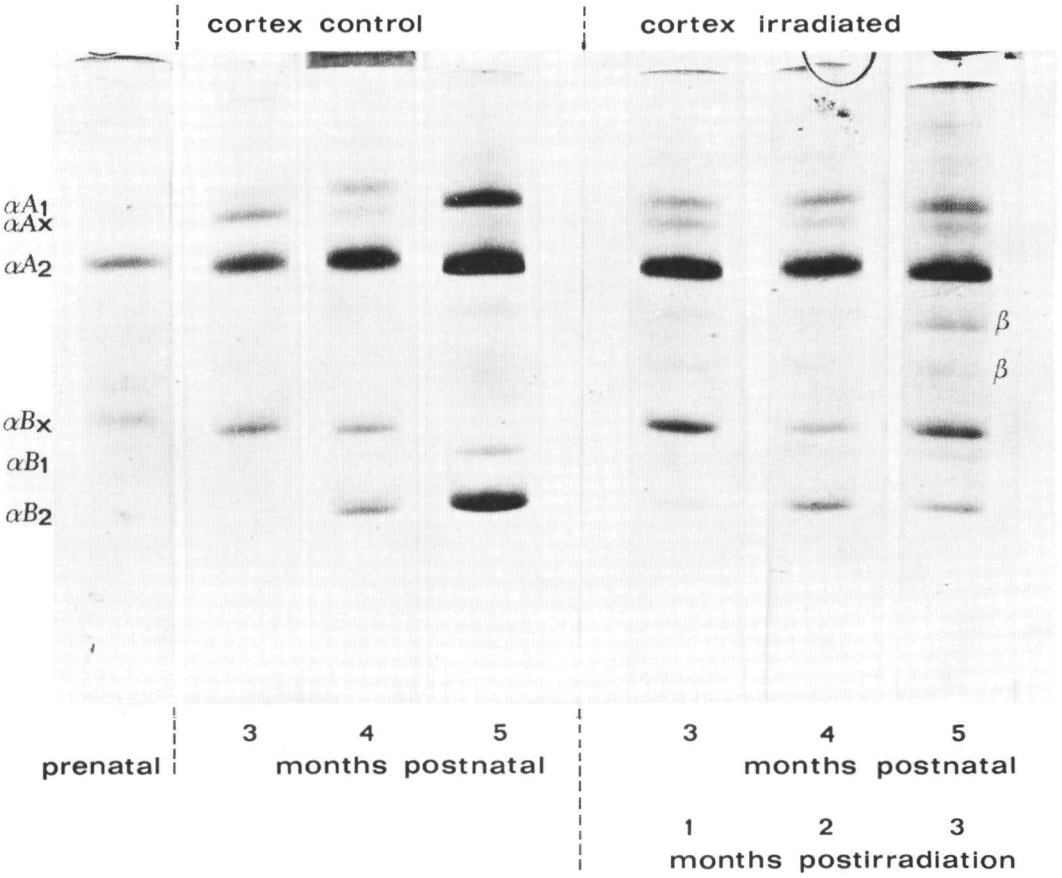

Fig. 2 Isoelectric focusing on polyacrylamide gels in the presence of 6M-urea of cortical α-crystallin from control and irradiated lenses. From left to right gel patterns of prenatal α-crystallin, three, four and five months postnatal, of the control as well as the irradiated lenses. With respect to the exposed lenses this corresponds with one, two and three months post-irradiation.

cortical α-crystallin from lenses two and three months after irradiation. They reveal five α-crystallin bands, and the latter in addition two β-crystallin bands.

Isoelectric focusing gel patterns of the α-crystallin of nuclear control and exposed lenses are shown in Fig. 3. Only quantitative differences were observed between the control and exposed lenses. A very complex pattern, partly due to β-crystallin chains, was obtained, three months after irradiation for both preparations.

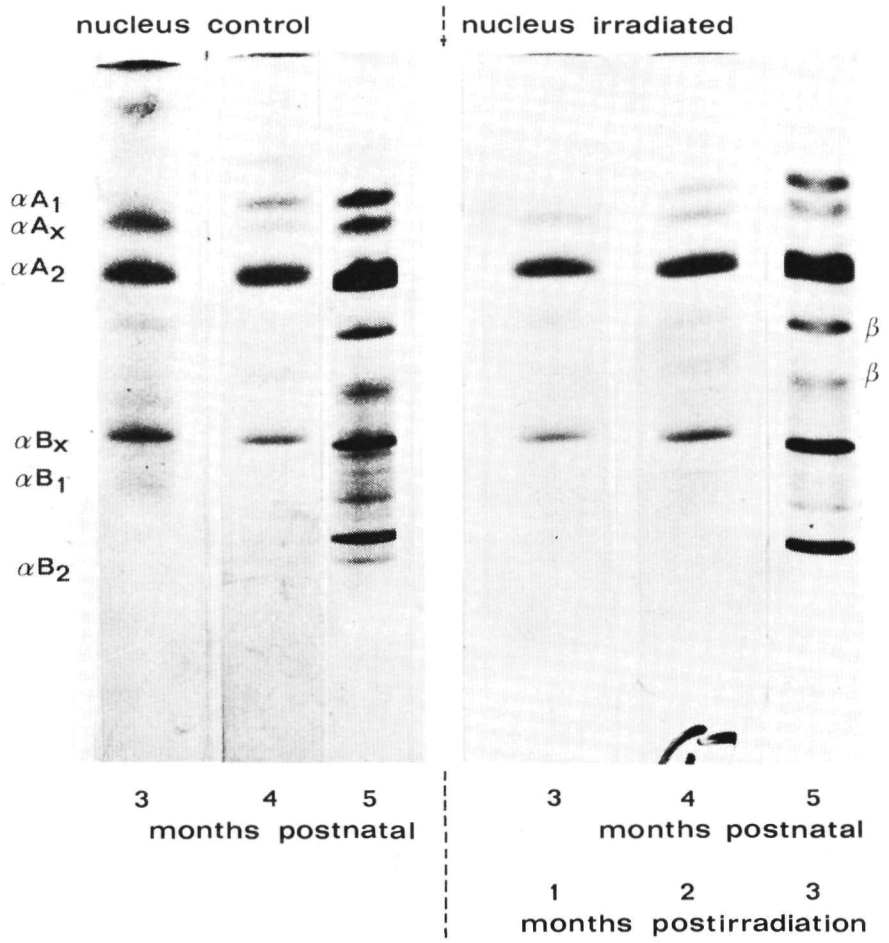

Fig. 3 Isoelectric focusing on polyacrylamide gels in the presence of 6M-urea of nuclear α-crystallin from control and irradiated lenses. From left to right gel patterns of α-crystallin from control and exposed lenses, three, four and five months old postnatal. In the case of the irradiated lenses this corresponds with one, two and three months after exposure.

4. Discussion

In the mature X-ray cataract, three months after irradiation, the thickness of the rabbit lens was considerably reduced compared with its control (Table I). This finding is consistent with ultrasonographic measurements on human senile cataract done by Goldmann and Favre, 1961; Goldmann and Niesel, 1964 and Babel, Psilas and Itin, 1969 (quoted from Weekers, Delmarcelle, Luyckx-Bacus and Collignon, 1973). A reduction of approximately 10% in wet weight of irradiated lenses was observed (Table I). This confirms earlier findings by van Heyningen, Pirie and Boag (1954). Most remarkable was the dramatic loss of protein, comparing irradiated lenses from two and three months after exposure.

In both, the control as well as the irradiated lenses, the percentages of water-soluble protein decrease, while those of water-insoluble protein increase between two and three months post-irradiation (Table II) . However, the magnitude of the changes differed markedly. The most pronounced changes were seen three months after irradiation. Here the content of water-insoluble protein greatly exceeded that of the water-soluble protein. These findings are in agreement with earlier observations on human lens proteins in relationship with aging and cataract (Mach, 1963; Pirie, 1968 and Clark et al., 1969).

This is also consistent with the findings of Rupe et al. (1958) and of Orekhovich et al. (1962) working with X-irradiated rabbit lenses. However, due to an erroneous calculation, the former authors arrived to a wrong conclusion from their experimental data.

The distribution of the crystallins as a function of cataract development was investigated. It turned out that marked changes are found only in the cataractous lens cortex, three months after irradiation; whereas for the other cortical and nuclear fractions similar patterns were observed as those from the control lenses (Table IV).

The relative increase of cortical HM-crystallin in mature cataract by about 40 fold (Table IV) and of total US by about tenfold (Table III) shows that in cataract the process of aggregation and insolubilization occurs at a much faster rate than under normal conditions. In this process HM-crystallin acts as an intermediate between the water-soluble proteins and the water-insoluble albumoid (Spector et al., 1971; Liem-The and Hoenders, 1974b; Liem-The et al., 1975b).

Mach (1963) observed that in comparison with normal lenses of the same age, there is a distinct diminution in the amount of total protein and of water-soluble protein with a concomitant increase in the amount of water-insoluble protein in human senile cataract. Sheridan and Zigman (1971) concluded from their study that conversion of soluble proteins to insoluble forms represents the manner in which soluble protein levels are depressed in the senile cataractous lenses, rather than leakage through the capsule. Croft (1973) suggested that γ-crystallin is transformed as a consequence of oxidation and subsequent insolubilization. As proposed by Harding (1972) at least a part of the missing γ-crystallin is converted into higher molecular weight protein. François and Rabaey (1959) and Mach (1963) using micro-electrophoresis on agar gel, and François et al. (1965) applying gel filtration technique, observed a decrease in the concentration of some proteins with low molecular weight in human cataractous lenses. Maisel and Goodman (1965) gave evidence that this was γ-crystallin. The possibility of leakage of these crystallins out of the lens by passage through the damaged capsule was supposed (Charlton and van Heyningen, 1968), but no direct evidence to support this idea could be found.

In the present paper, evidence is presented that a portion of the soluble protein has leaked out (Table I and VI) and another part became insoluble (Table II and III). Proof for the loss of protein is the decrease of total protein in exposed lenses, two to three months post-irradiation. Our data at present strongly suggest that not only γ-crystallin but also β-crystallin is lost in considerable amounts from the soluble protein fraction. So, this points to the fact that preferentially the crystallins with lower molecular weights are lost.

A marked decrease in the concentration of β-crystallin and only to some extent of γ-crystallin was seen three months after irradiation (Table IV, Fig. 1). The explanation

is that β-crystallins are lost in two ways, first becoming insoluble and second by leakage, whereas γ-crystallin is primarily lost by leakage

Earlier observations have shown that changes in the subunit structure of α-crystallin occur during the development from prenatal to the adult bovine lens (Rabaey, 1965, Mehta and Maisel, 1968, Schoenmakers and Bloemendal, 1968, Palmer and Papaconstantinou, 1968, van Kamp, Struyker Boudier and Hoenders, 1974) Recent experiments have shown that αA_X and αB_X found in bovine prenatal α-crystallin (van Kamp et al., 1974) are shortened αA_2 and αB_2 chains, respectively (van Kleef, 1975) Degraded and deamidated α crystallin subunits were observed before in the nucleus of the adult bovine lens (van Kleef, Nijzink-Maas and Hoenders, 1974) Transformation of α-crystallin polypeptides from the nuclear part of bovine lenses were also observed by Stauffer, Rothschild, Wandel and Spector (1974)

From the present report it appears that similar alterations take place in non-irradiated rabbit lenses, as also observed by SDS gel electrophoresis In the cortex of the three-month-old non-exposed lens initially the degraded A_X chain was formed from the *de novo* synthesized polypeptide A_2 and later on (four and five months old), by deamidation of A_2, the A_1 chain As for the B chains, a completely other course of post-synthetical processing is going on Initially the *de novo* synthesized B_2 chain obviously is converted into B_X completely In the four-month-old non-irradiated animal B_2 is only partly converted Five months after birth B_2 preponderates, whereas B_X has disappeared and some B_1 has been formed

At this moment no further explanation for these age-related changes can be given

In the nucleus of the control lens essentially the same processes occur A difference with regard to the cortex is the fact that polypeptide chains of β-crystallin are clearly present in the α-crystallin preparation

Comparison of the α-crystallins from control and irradiated cortices reveals that the switch of post-synthetic processes between the age of four and five months does not take place in the irradiated cortex Furthermore, β-crystallin peptides have appeared three months after irradiation, thus causing great similarity with respect to the corresponding nuclear preparations This suggests that X-irradiation results in an early aging of the cortical α-crystallin

A comparison of the polypeptide chain composition of the α-crystallins from the cortex and nucleus of the control lenses showed, besides the presence of β-crystallin chains, a considerable difference only at the age of five months In the nuclear α-crystallin A_X and B_X were clearly present, in the cortical α-crystallin they were absent It looks as if after the fourth month post-synthetic processes in the nucleus take place, which have been switched off in the cortex

It seems worthwhile to gather more information about post-synthetic processes, because from this knowledge more fundamental data with respect to aging processes as well as cataract formation should arise

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Chapter 7

Characterization of Crystallins in Normal and Cataractous Human Eye Lens

SUMMARY

The cortical and nuclear soluble proteins of aged normal and cataractous lenses were separated according to their molecular size on agarose Biogel A-5m/A-0.5m; essentially four components were obtained. The high-molecular weight (HM-)crystallin was found in relatively small amounts in the nuclear part of the cataractous lenses. Furthermore, α-crystallin was virtually absent in the soluble fraction of the normal and cataractous lens nucleus. Electron micrographs of HM- and α-crystallin revealed the presence of irregularly shaped giant aggregates with sizes up to 500 nm for the former, and spheres of approximately 18.5 nm for the latter. UV spectra of the HM-, α-, β- and γ-crystallins from older normal and senile cataractous lenses were similar, the same held true for their sedimentation coefficients. Applying isoelectric focusing on thin layer plates, α-crystallin revealed a diffuse region at about pH 5, whereas at least 12 bands were found for β-crystallin and about 14 bands for γ-crystallin both between pH 5 and 7. It was remarkable that numerous bands, especially in the acidic region, had the same isoelectric points in β- and γ-crystallin. This held for the cortical and nuclear parts of normal and cataractous human lens.

Upon isoelectric focusing in the presence of 6M-urea, the subunits of all the crystallins showed isoelectric points mainly between pH 5.5 and 7.0. HM- and α-crystallin revealed similar patterns with at least 8 bands; β- and γ-crsytallin showed each at least 10 bands. Six bands in the β- and γ-crystallin patterns revealed identical isoelectric points. Except for quantitative differences, the isofocusing patterns of the crystallins in cortical and nuclear aged normal and cataractous lenses were the same.

Molecular weight estimation by gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) revealed for HM-crystallin a pattern with about 9 bands corresponding with molecular weights between 11 000 and 50 000 Daltons. Alpha-crystallin showed 4 bands, three heavy and one light stained, corresponding with molecular weights from 17 500 to 22 000 Daltons. For β-crystallin 7 polypeptide chains were found having molecular weights from 18 500 to about 50 000 Daltons. Gamma-crystallin showed a pattern with seven bands corresponding with molecular weights from 11 000 to about 50 000 Daltons.

Immunoelectrophoretically β- and γ-crystallin had a main precipitin line with the *same* mobility, in addition to their own characteristic precipitin lines. Applying immunodiffusion technique it was shown that γ-crystallin contained some β-crystallin determinants. Purification by rechromatography of β- and γ-crystallin revealed no differences in the immunochemical results.

On isoelectric focusing in the presence of 6M-urea the urea-soluble (US) fraction revealed numerous subunits, which were also found in α-crystallin, as well as in β- and/or γ-crystallin. In addition, acidic subunits with isoelectric points between pH 4.7 and 5.4 were observed. Essentially the same patterns were found for the US fraction of cortical and nuclear parts of normal and cataractous lenses. By SDS gel electrophoresis again a pattern composed of α-, β- and γ-crystallin subunits was found.

1. Introduction

In the report on the National Eye Institute Cataract Workshop, Spector (1974) stated that cataract is a major cause of visual disability and blindness throughout the world. "It was urged that more extensive research on the cataract problem is needed".

The classification of different types of cataract is a problem. Some investigators have made a classification of human senile cataracts based on the location of the opacity (a.o. Mach, 1963; Maraini and Pescatori, 1972; Nordmann, 1972 and Friedburg, 1973). Pirie (1968) has classified senile cataracts on the basis of increasing nuclear pigmentation, while Clark, Zigman and Lerman (1969) have classified them only by age. For a good review see Barber (1973).

We have made an attempt to classify the senile cataracts, before investigating the protein chemical aspects, based on a combination of the location and intensity of the opacity, color and age.

Another prerequisite is of course a proper characterization of the normal human lens proteins. Maraini and Mangili (1973), applying polyacrylamide gel electrophoresis in the presence of urea, have shown that the polypeptide chain composition of α-crystallin is similar in normal and cataractous human lenses. Recently, Spector, Stauffer and Sigelman (1973) have determined some characteristics of the normal human lens crystallins.

In senile cataractous lenses a marked increase in the water-insoluble fraction with a concomitant decrease in total protein and water-soluble protein content was observed amongst others by Mach (1963). Studies on the soluble protein fraction applying electrophoretic and chromatographic methods have shown that in cataractous lenses, especially the advanced ones, the low molecular weight crystallins have decreased in content (a.o. Tapaszto, 1962; Mach, 1963; Malik, Gupta, Chatterji and Agarwal, 1969; François, Rabaey and Stockmans, 1965; Charlton and van Heyningen, 1968; François, Rabaey and and Boyen-Rikkers, 1969; Clark et al., 1969). The disappearance of soluble protein may be attributed to leakage of the low molecular weight proteins (Charlton and van Heyningen, 1968; Mach, 1963) and/or to insolubilization processes (Clark et al., 1969; Harding, 1972).

Investigation of the insoluble protein fraction of senile cataractous lenses by amino acid analysis, electrophoretic and immunochemical methods, has shown that it is composed mainly of α-crystallin, and probably also minor amounts of β- and γ-crystallin. (Clark et al., 1969). It has been suggested that one of the mechanisms of insolubilization of soluble protein might be oxidation of protein sulfhydryl groups. However, Pirie (1968) and Harding (1969, 1970, 1972) found that this oxidation of protein SH-groups only occurs during aerobic extraction procedures. According to Harding these reactions are due to higher reactivity of SH-groups, presumably caused by unfolding of the tertiary structure of the soluble protein. So at least part of the insoluble protein is an artefact; according to Pirie (1968) some other part may be insoluble as a result of photo-oxidative modification leading to cross-linking by quinoid molecules.

In the present investigation a characterization was made of the structural lens proteins in aged normal human lenses. Furthermore, differences between normal and cataractous lenses were sought for. The cataractous lenses used in this study have opacities in both the nucleus and cortex, moreover the color of the nuclei was yellow or light brown. Various techniques have been applied, i.e. molecular sieving, isoelectric focusing (in the presence and absence of urea), polyacrylamide gel electrophoresis in the presence of SDS, immunochemical methods, electron microscopy, sedimentation analysis and UV absorption spectrophotometry.

2. Materials and Methods

The classification of human senile cataract is depicted in Table I.

Table I

Classification of human senile cataract

a) Hypermature cataract	Morgagni
	shriveled
b) Capsular cataract	anterior
	posterior
	anterior-posterior
c) Cortical cataract	waterclefts
	opacities – anterior
	posterior
	anterior-posterior
	intensity of opacification: +, ++, +++.
d) Nuclear cataract	color – yellow
	brownish
	deep brown
	nigra (black)
	intensity of opacification: +, ++.

Lenses

Normal lenses were donated by Prof. Dr. W.J. van Doorenmaalen, Department of Anatomy and Embryology, State University Utrecht, Utrecht and by ophthalmologist T.T. Tjan, Eye Clinic of the Wilhelmina Gasthuis Hospital, Amsterdam.

Senile cataractous lenses were a gift from Prof. Dr. J. Wollensak, Eye Clinic, Free University, West-Berlin. Immediately after surgery they were stored at -70°C. Transport to Nijmegen took place by the use of a Dewar container, partly filled with dry ice.

Normal completely transparent lenses from 60 to 80 year old were used; the lens nuclei were soft of consistency and apparently had not undergone sclerosis.

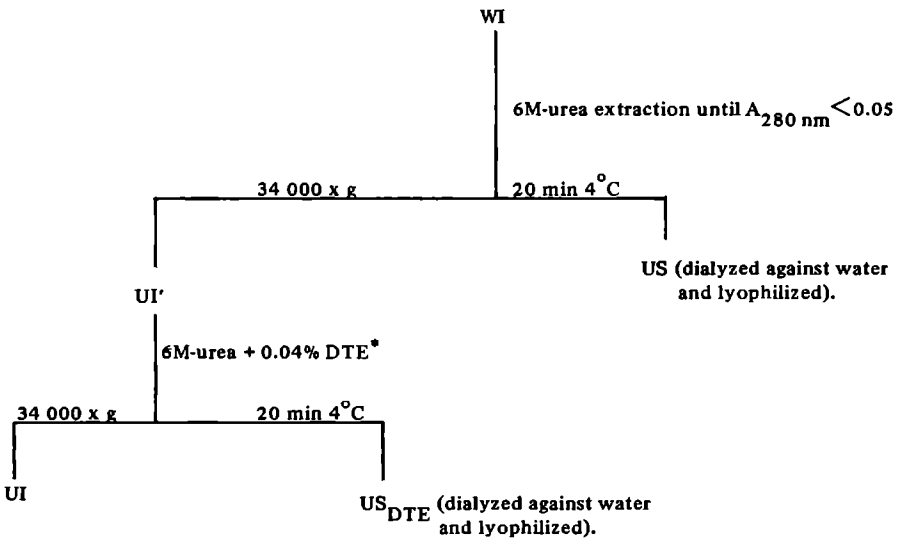
Cataractous lenses with both opaque hardened nuclei, yellow or light brown colored, and cortices showing opacities to some degree were used [a combination of c) and d) in Table I]. In these experiments the normal and cataractous lenses were of the same age.

Preparation and gel chromatography of lens extract

Outer cortical and inner nuclear lens parts (40% and 20% of the lens wet weight, respectively) were separately fractionated. Four to ten lenses in age ranging from 60-70

years old or from 70-80 years old were used for each fractionation. Lens material was homogenized by stirring under nitrogen overnight at 4°C in phosphate-saline buffer pH 7.3 (0.15 M NaCl/0.02 M phosphate). The lens extract was obtained by centrifuging at 10 000 rpm (7850 x g) in a SM 24 rotor at 4°C for 15 min. Fractionation was performed using Agarose Biogel A-5m/A-0.5m. (for further details see Liem-The and Hoenders, 1974 b).

In the case of human lenses, the water-insoluble (WI) fraction was fractionated into three fractions, as follows:



* DTE = 1,4-dithioerythritol

Sedimentation analysis

The sedimentation coefficient of fractions with the highest concentration of the crystallin peaks obtained by gel chromatography was determined as described previously (Liem-The and Hoenders, 1974a).

Electron microscopy

Electron micrographs of α- and HM-crystallin from the cortex and nucleus of normal and cataractous lenses were taken as described earlier (Liem-The, Stols and Hoenders, 1975a).

Immunochemical analyses

HM-, α-, β-, γ-crystallin and total lens extract were subjected to immunoelectrophoresis according to the micromodification of Scheidegger (1955). Double diffusion

precipitin tests were performed by the method of Ouchterlony (1958). Antisera against human total lens proteins and against calf β- and γ-crystallin were raised in white New Zealand rabbits.

Isoelectric focusing on thin-layer polyacrylamide gels

The method according to Bours (1971) was used.

Isoelectric focusing in the presence of 6M-urea

The method used was as described previously (Liem-The and Hoenders, 1974b), using Ampholine pH 3-10 and pH 5-8.

Gel electrophoresis in the presence of sodium dodecyl sulphate (SDS)

The method was carried out as described earlier (Liem-The and Hoenders, 1974b).

3. Results

Column chromatography

The elution patterns of lens extracts of the cortex and nucleus of the normal and cataractous lenses are depicted in Fig. 1. They revealed essentially four peaks, HM-, α-, β- and γ-crystallin. In the nuclear fractions from cataractous lenses, HM-crystallin was found in relatively small amounts.

Sedimentation analysis

The various crystallins from the cortical and nuclear part of normal and cataractous lenses were submitted once or twice to sedimentation in the analytical ultracentrifuge. The different preparations of each type of crystallin did not reveal significant differences with respect to their sedimentation coefficients. The following values were obtained: approximately 50 S for HM-crystallin; 18.2 ± 0.5 S for α-crystallin; β-crystallin was heterogeneous and revealed essentially two values, i.e. 9.0 ± 0.4 S and 5.6 ± 0.3 S; 2.1 ± 0.1 S for γ-crystallin.

UV absorption spectra

UV absorption spectra of the human crystallins are similar to those found in calf (van Kamp and Hoenders, 1973) and rabbit lenses (Liem-The, Stols and Hoenders, 1975a). The same spectra were found for the corresponding crystallins from the normal and cataractous lens cortex and nucleus.

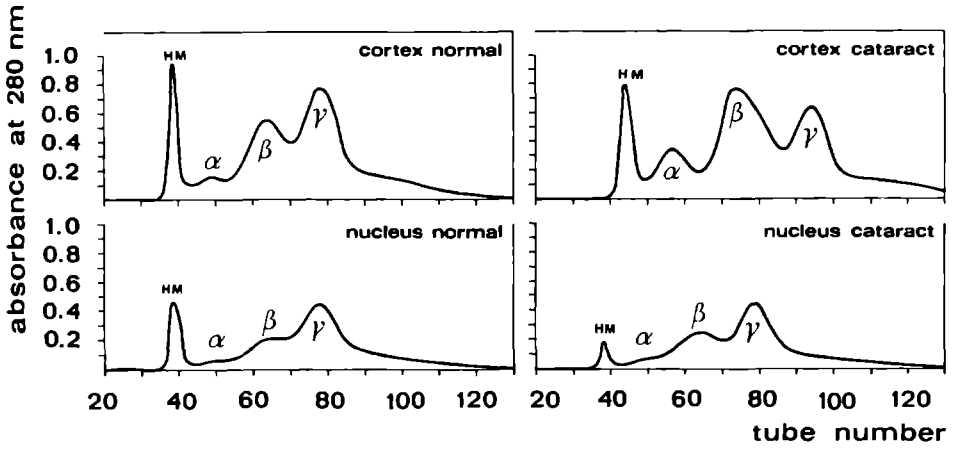


Fig. 1 Elution patterns obtained by gel chromatography on Biogel A-5m/A-0.5m of soluble proteins from cortical and nuclear parts of the normal and cataractous human lens The distribution as percentages of the crystallins in the lens-extracts obtained by planimetry of the elution patterns were as follows.

		HM	α	β	γ
normal	cortex	11 0	6.3	30.5	52 2
	nucleus	10 2	3.6	21.0	65 3
cataract	cortex	9.5	17.0	34 8	38 8
	nucleus	4.2	3.3	30.7	61.8

Electron microscopy

Electron micrographs of normal cortex α-crystallin showed globular molecules with a diameter of approximately 18.5 nm, similar to those found in rabbit lenses (Liem-The, Stols and Hoenders, 1975a) and calf lenses (Kramps, Stols, Hoenders and de Groot, 1975). The same spheres were observed for α-crystallin of normal and cataractous lens nucleus and cortex. An electron micrograph of HM-crystallin from normal cortex is shown in Fig. 2. Irregularly shaped particles of varying sizes up to 500 nm were observed. These particles were also seen in electron micrographs of cataractous cortex and nucleus, as well as normal nucleus HM-crystallin.

Immunochemical analysis

Immunoelectrophoresis revealed similar patterns in normal and cataractous lens fractions (Fig. 3). In HM- and α-crystallin a great proportion of the material seemed to be high molecular because it tended to precipitate near the well. HM-crystallin showed a precipitin arc with the same mobility as α-crystallin. Beta- and γ-crystallin had a main precipitin line with virtually the same mobility, in addition both had their own charac-

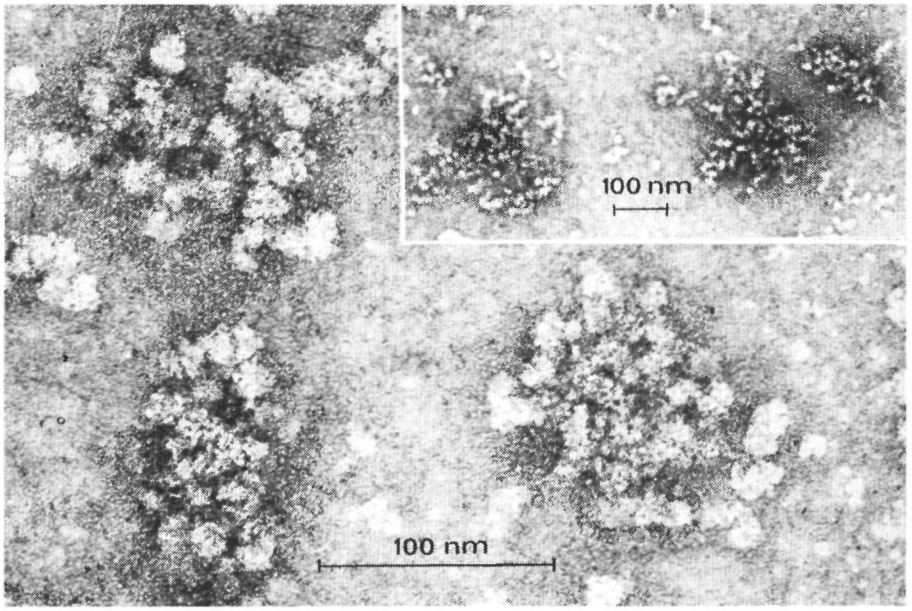


Fig. 2 Electron micrograph of HM-crystallin from cortex of the human normal lens. It shows irregularly shaped, large aggregates.

teristic lines too, although in minor amounts. For example, γ-crystallin had at least one additional line in the region towards the kathode. To be sure that β- and γ-crystallin were not contaminated with each other, they were subjected to rechromatography. Immunoelectrophoresis of these rechromatographed samples again showed a main precipitin line of the same mobility.

On immunodiffusion β- and γ-crystallin showed a reaction of complete identity for two antigens, expressed by two precipitin lines, one heavy and one very faint. Furthermore, β-crystallin appeared to have one additional line which was specific for β-crystallin and two additional precipitin lines were observed to be specific for γ-crystallin (Fig. 4a).

Against anti-calf β-crystallin, human β- and γ-crystallin revealed one precipitin line which fused completely. In addition, one specific β-crystallin antigen was found (Fig. 4b).

Anti-calf γ-crystallin was found to react only with human γ-crystallin; no precipitation was observed against β-crystallin (Fig. 4c).

Isoelectric focusing on thin-layer polyacrylamide gels

The patterns obtained by isoelectric focusing on thin layer plates are presented in Fig. 5. For comparison, the total lens extract and the crystallins of the rabbit were included. HM-crystallin could not be re-dissolved in water after lyophilization, therefore its pattern was not included. Alpha-crystallin revealed a diffuse region at about pH 5, while between pH 5.3 and 7.2 at least 12 and about 14 bands were found for β- and γ-crystallin, respectively. Remarkable is that numerous bands, especially in the acidic region, have the same isoelectric points in β- and γ-crystallin. Rechromatographed

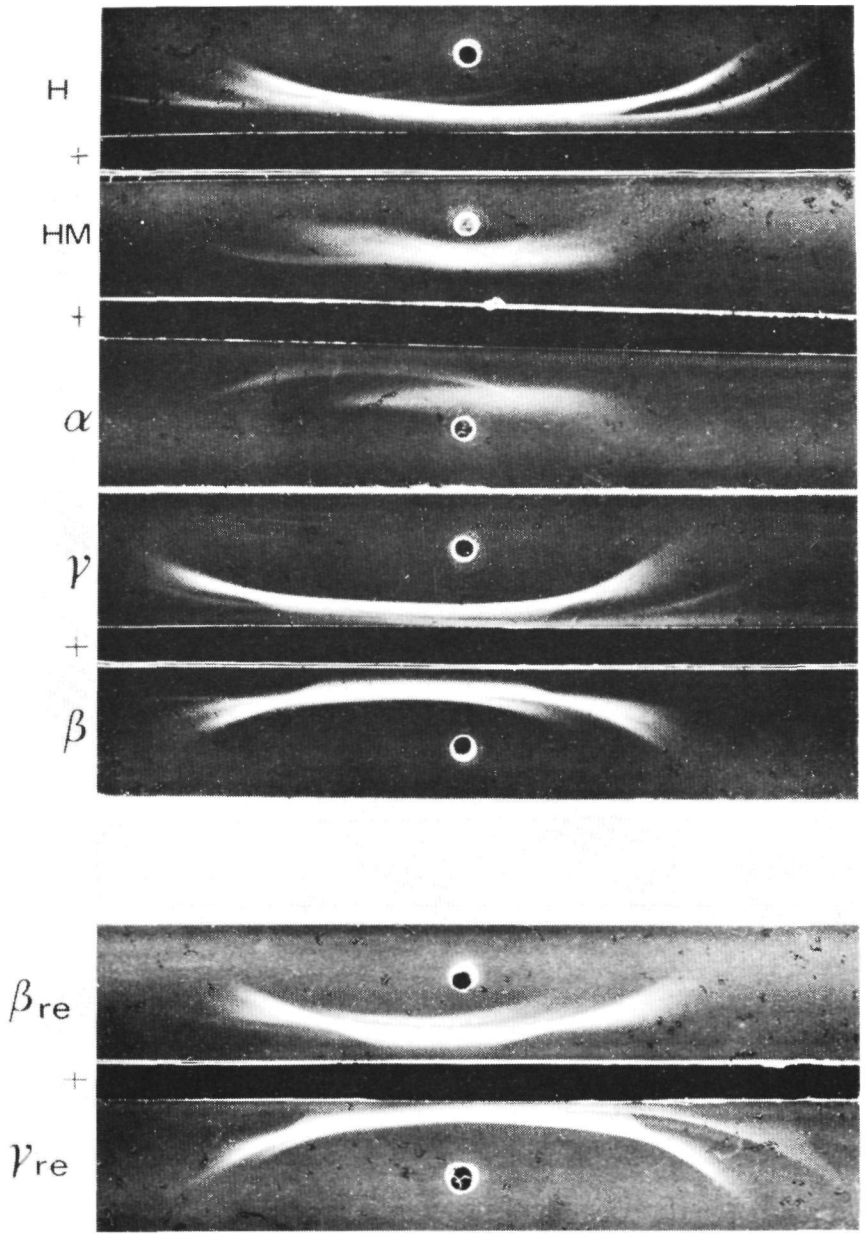


Fig. 3 Immunoelectrophoresis of human crystallins against anti-human lens serum. H = human lens extract. $Beta_{re}$ and γ_{re} stands for rechromatographed β- and γ-crystallin.

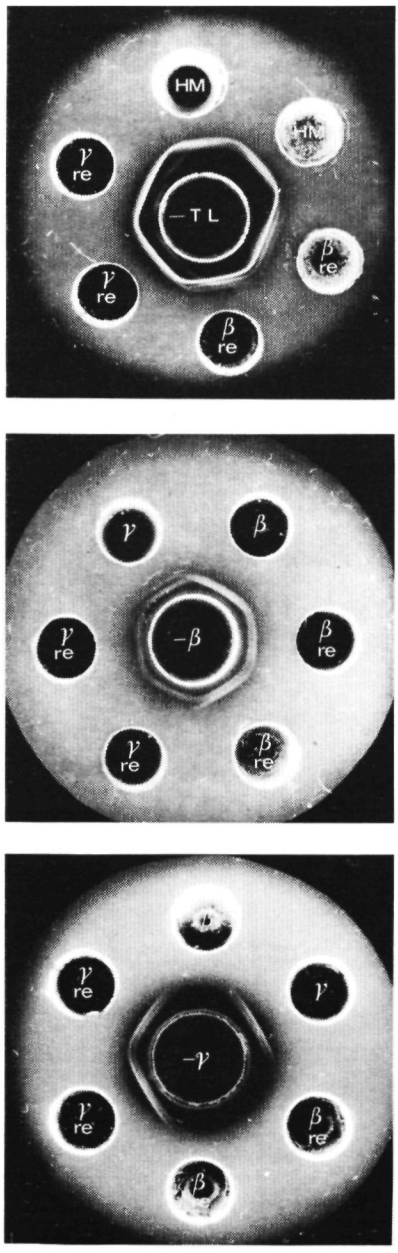


Fig. 4a Immunodiffusion patterns of rechromatographed β- and γ-crystallin and of HM-crystallin against anti-human lens serum.

4b Immunodiffusion patterns of β- and γ-crystallin, as such and rechromatographed, against anti-calf β-crystallin.

4c Immunodiffusion patterns of β- and γ-crystallin, as such and rechromatographed, against anti-calf γ-crystallin.

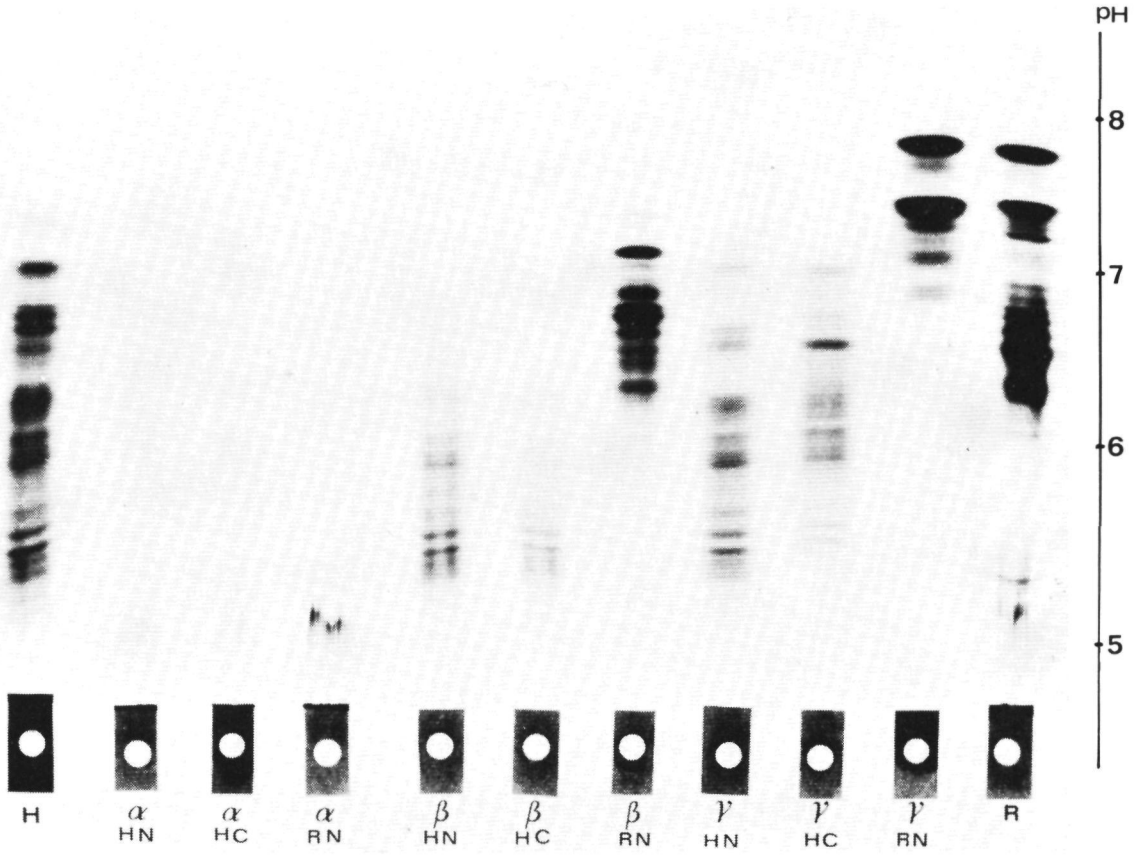


Fig. 5 Patterns of soluble protein fractions of human and rabbit lenses obtained by isoelectric focusing on thin layer plates. H = human whole lens extract; R = rabbit whole lens extract; HN = human normal; HC = human cataract and RN = rabbit normal.

human β- and γ-crystallin showed the same results. Except for considerable quantitative differences, this holds for the cortical and nuclear normal and cataractous human lens crystallins.

A comparison of human and rabbit β-crystallin showed that the isoelectric points of the former are lower than those of the latter. The same applies for γ-crystallin of human and rabbit lens.

Isoelectric focusing in the presence of 6M-urea

The gel patterns obtained by this technique are shown in Fig. 6a. The subunits of all the crystallins, HM-, α-, β- as well as γ-crystallin, have isoelectric points between pH

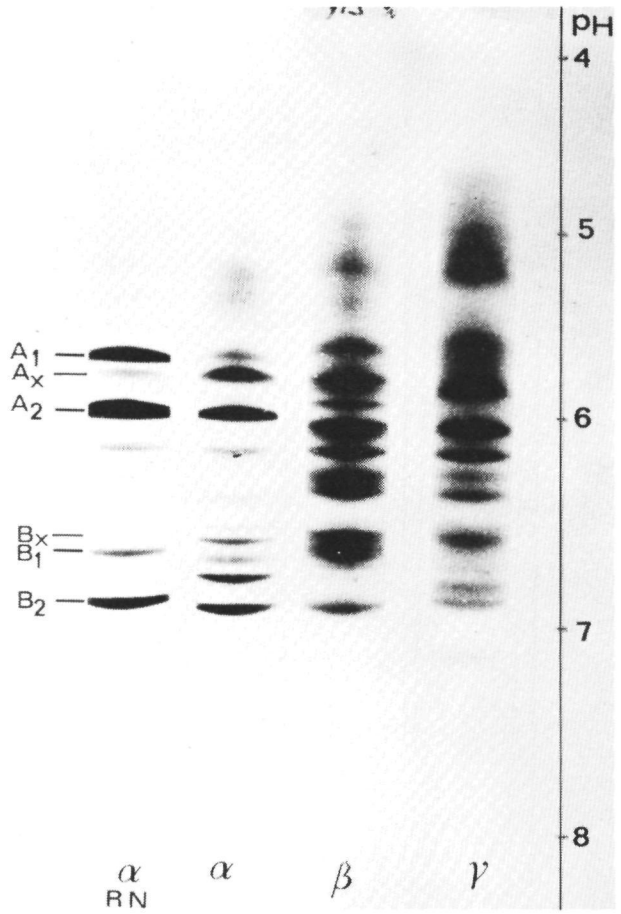
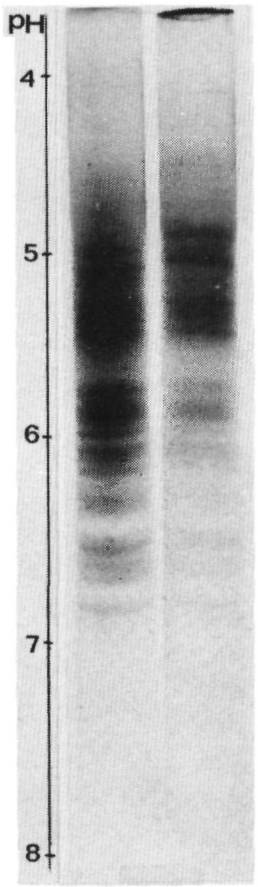


Fig. 6a Isoelectric focusing on polyacrylamide gels in the presence of 6M-urea and 0.04% DTE. Acrylamide concentration was 7.5% (w/v) and Ampholine pH 3-10 was used. From left to right gel patterns of α-crystallin from normal rabbit lens cortex (RN), α-, β- and γ-crystallin of the cortical part of cataractous human lens.



4.7 and 7.1. Remarkable are the bands observed at pH range 4.7 to 5.4, which are not found in rabbit lens. HM- and α-crystallin revealed similar patterns with at least 8 bands; for β- and γ-crystallin at least 10 bands were found. About 6 bands in the β- and γ-crystallin patterns showed identical isoelectric points. Except for quantitative differences, the isofocusing patterns of the crystallins in cortical and nuclear aged normal and cataractous lenses were the same.

In Fig. 6b the patterns of the urea-soluble (US) fractions, extracted in the absence and presence of DTE, are depicted. Here the acidic bands in the region between pH 4.7 and 5.4 are remarkable. Especially the US fraction extracted with DTE consisted for the greater part of these acidic chains.

Also worthwhile mentioning is the fact that on top of the gel a dark rim is found after staining, indicating that some protein did not penetrate the gel, although the acrylamide concentration used in this case was only 3.5% (w/v).

Fig. 6b Isoelectric focusing gel patterns. Acrylamide concentration was 3.5% (w/v) and Ampholine pH 3-10 was used. On the left, the pattern of US fraction and on the right that of US fraction extracted in the presence of 0.04% DTE. Both samples were isolated from the cortical part of normal human lens.

Gel electrophoresis in the presence of SDS

Molecular weight estimations by SDS gel electrophoresis are presented in Fig. 7 and Table II. Alpha-crystallin revealed three main bands corresponding with molecular weights between 18 000 and 22 000 Daltons; and often trace amounts of a chain of 17 500 Daltons. Beta-crystallin showed 7 bands corresponding with polypeptides of 18 500 to about 50 000 Daltons. Gamma-crystallin has 7 polypeptide chains between 11 000 and about 50 000 Daltons. HM-crystallin revealed 9 polypeptides between

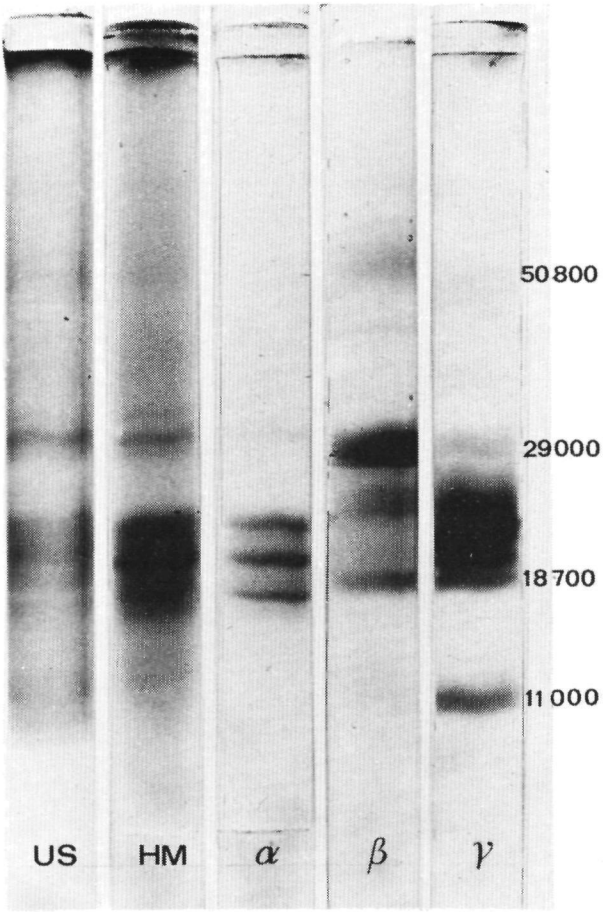


Fig. 7 Polyacrylamide gel electrophoresis in the presence of SDS. From left to right gel patterns of US fraction, HM-, α-, β- and γ-crystallin from the cortex of cataractous human lens.

11 000 and 50 000 Daltons; a similar pattern was found for US fraction, though an additional chain, $\leqslant$ 10 000 Daltons, was also seen. Quantitative differences of the chains were found for cortical and nuclear fractions. The patterns from normal and cataractous lenses revealed no significant differences. It should not be overlooked that in HM-crystallin and US fraction a considerable part of the sample did not penetrate into the gel.

Table II

Molecular weights of polypeptide chains from human lens crystallins, as estimated by gel electrophoresis in the presence of SDS

US	HM	α	β	γ
(51 800)	49 800		50 800	50 800
			41 300	
(30 900)	31 000			
29 300	29 600		29 200	29 000
(26 000)			26 300	
			23 800	
			22 700	22 700
21 900	22 000	21 900		
	21 200			21 300
20 200	20 400	20 100		19 700
			18 600	18 700
18 300	18 300	18 200		
(17 500)	17 400	(17 500)		
(11 000)	(11 000)			11 000
(≤ 10 000)				

Values of minor components are given in parenthesis.
Mean values of three to six estimations.

4. Discussion

In the present study only quantitative differences has been found as far in normal and cataractous human lens crystallins of the same age. This is consistent with earlier observations by François, Wieme, Rabaey and Neetens (1954) who found no differences in the electrophoretic behaviour between the soluble proteins of normal and cataractous lenses of aged humans. Clark et al. (1969) found that senile normal lenses and senile cataracts resemble each other as judged by their amino acid analyses, extinction coefficients and sulfhydryl contents. Recently, York, Martinez and Manski (1972) showed immunochemically a similarity in patterns for normal and cataractous human lenses; Maraini and Mangili (1973) investigating the α-crystallin of normal and cataractous lenses by molecular sieving techniques and urea gels, observed the same patterns for both. By electron microscopy HM-crystallin showed particles which seemed to be aggregates mainly composed of α-crystallin molecules (Fig. 2). Its polypeptide chain composition, as well as that of the urea-soluble fraction, revealed the presence of chains from α- and β- and minor amounts of γ-crystallin (Fig. 7 and Table II). Thus,

judging from these findings, it seems reasonable to assume that HM-crystallin in human lenses, like in other mammals (cow and rabbit, Spector, Freund, Li and Augusteyn, 1971 and Liem-The and Hoenders, 1974b, respectively), is an intermediate in the insolubilization process. From amino acid analyses Clark et al (1969) obtained the indication that α-crystallin and albumoid of the human lens compare closely, variation in their data suggested, however, that albumoid contains some β- and γ-crystallin as well.

Another interesting aspect is the presence of acidic chains in the US fraction, as seen on isoelectric focusing in the presence of urea between pH 4 7 and 5.4 (Fig. 6b) These chains, though in smaller amounts, are also found in the soluble proteins, especially β- and γ-crystallin (Fig 6a). One might suppose that they are formed by post-synthetic processes, like those found in α-crystallin. Stauffer, Rothschild, Wandel and Spector (1974) found transformations resulting in the appearance of new acidic chains in α-crystallin from old bovine lenses. Van Kamp, Schats and Hoenders, 1973, van Kleef (1975) and Liem-The, Ringens, Holtslag and Hoenders (1975) observed changes in the subunit composition of α-crystallin due to aging processes in cow and rabbit lenses There are indications that these processes include deamidation and partial degradation By analogy it is tempting to suppose that the acidic chains in the protein fractions of the human lenses are products of similar aging processes. In this investigation all lens material used was from elderly humans, which makes this assumption of aging processes more acceptable.

The characteristics for human γ-crystallin deviate strongly from the generally accepted characteristics of γ-crystallin from other mammals For instance, the isoelectric points of its chains range from pH 5 to 7, unlike those of γ-crystallin from cow or rabbit, which are between pH 7 and 8 (Fig. 5) On immunochemical analysis human γ-crystallin showed specific γ-crystallin determinants, beside the presence of β-crystallin determinants Furthermore, based upon isoelectric focusing (Fig 6a) and SDS gel electrophoresis (Fig. 7) its subunit composition appears to be partially identical to that of β-crystallin. On the other hand, by gel chromatography γ-crystallin was eluted at a similar volume as that of other species, its UV absorption spectra and sedimentation coefficient are also typical for γ-crystallin Thus, it seems that γ-crystallin chains of normal as well as cataractous human lenses have isoelectric points more acidic than those found for calf and rabbit γ-crystallin chains Kabasawa and Kinoshita (1974) found that older bovine lens contains two forms of γ-crystallin one γ which is identical with calf lens is found in the nucleus, the second γ is found in the cortex and has a higher molecular weight and lower isoelectric point than that of the calf lens In addition, the present results point to the occurrence of low molecular weight β-crystallin, which is of about the same size as γ-crystallin. This finding confirms the earlier observations by van Dam (1966), Croft (1973) and Kabasawa and Kinoshita (1974), they found in old bovine lens a low molecular weight β-crystallin (β_S) Croft (1973) did not find any γ-crystallin, but only low molecular weight β-crystallin in cataractous human lens.

According to many authors (a o. Mach, 1963, François 1965, François et al. 1969, Charlton and van Heyningen, 1968 and many others) a decrease in the proportion of γ-crystallin was observed in human senile cataractous lens, especially in the advanced ones In the present study this phenomenon was seen only in the cortical part of the cataractous lens (Fig. 1)

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Summary and Concluding Remarks

The aim of investigations described in this thesis was to study possible changes in the composition and subunit structure of the eye lens proteins as a consequence of cataract formation. For this purpose unilateral, X-ray induced, cataract in rabbit lenses was chosen as a model system.

A prerequisite is the thorough knowledge of the properties of normal lens proteins. Therefore, a characterization was made of the rabbit lens crystallins (chapter 2). The presence of a high molecular weight protein fraction (HM-crystallin) besides one α-, three β- and one γ-crystallin fraction was observed. HM-crystallin showed a subunit composition similar to that of α-crystallin.

Earlier data in literature suggested that the albumoid (water-insoluble fraction), which can be subdivided into a urea-soluble (US), and a urea-insoluble (UI) fraction consisting of membrane fragments, is derived from α-crystallin. It is known that this albumoid is found in great amounts in cataractous lenses. In order to verify this relationship, the subunit composition of α-crystallin and of the US fraction was investigated (chapter 3). As starting materials, the outer lens cortex (young cells, 40% of wet weight) and the lens nucleus (old cells, 20% of wet weight) were used. The results obtained showed that cortical α-crystallin possessed a relatively simple polypeptide chain composition. Nuclear α-crystallin revealed a more complex composition. This complexity seemed to be partly caused by age-related deamidation and partial degradation processes. Furthermore it is due to the presence of minor amounts of polypeptide chains of β-crystallin. Similar peptide patterns as in α-crystallin were found for HM-crystallin and US fraction from the cortex and the nucleus of the lens. On account of these similarities, it was suggested that HM-crystallin is an intermediate in the insolubilization process. Further support for this suggestion was the electron microscopic finding that HM-crystallin represents a population of aggregates with sizes up to 500 nm (chapter 4). Moreover, ion exchange chromatography of HM-crystallin revealed that these giant aggregates could be broken down and separated into α- and β-crystallins with sedimentation coefficients of 20 S and 4 S, respectively. This in turn gave rise to the assumption of how these two types of crystallins might be arranged in the HM-crystallin aggregates.

In chapter 5 a description is given of the subunit composition of α-, HM-crystallin and US fraction prepared from X-ray induced mature cataractous lenses. The findings obtained showed that in these fractions the polypeptide chain composition is becoming more complex, going from α-crystallin via HM-crystallin to US fraction, thus resulting in similar compositions as found in the corresponding fractions from the normal nucleus. These fractions were composed of α- and, in gradually increasing amounts, of β-crystallin peptide chains in the case of cataractous α- and HM-crystallin; as for US fraction also minor amounts of γ-crystallin chains were involved. This held for the cortical as well as the nuclear lens fractions. It was striking that HM-crystallin of the cataractous cortex already showed a complex composition, which normally is only found in that of the nucleus. In addition, the HM-crystallin content of the cataractous cortex is very high, which again could be found only in the nucleus of the control lens. Electron micrographs showed the presence of very large aggregates for HM-crystallin in cataractous cortex in contrast with normal cortical HM-crystallin, which was shown to contain relatively small aggregates. All these findings strongly suggest that in lenses with mature cataract aging processes have taken place, progressively.

In chapter 6 a description is given of the development of aggregation and insolubilization of the crystallins one, two and three months after X-irradiation. Marked changes became manifest only three months after exposure (mature cataract). The HM-crystallin content, especially in the cortex, increased dramatically as compared to that of the control lens. As for the US fraction, a tenfold increase in comparison with that of the control was observed. Furthermore, proof of loss of protein was a decrease of total protein found in lenses between two and three months after irradiation. The loss is reflected especially in the disappearance of β-crystallin (55%) and γ-crystallin (65%).

Age-related, post-synthetic processes appeared to have taken place in α-crystallin from the control lenses, as reflected in the alterations of the proportions of degraded and deamidated chains. In the case of the irradiated lens, cortical α-crystallin revealed a subunit composition similar to that of nuclear α-crystallin preparations. Thus, this apparently early aging observed for α-crystallin in young cells and the excessive increase of albumoid are further evidence that in X-ray induced cataract events are taking place, which resemble accelerated aging processes.

In chapter 7 the cataracteristics of the crystallins from aged normal and senile cataractous human lenses are described. Fractionation of the soluble proteins by gel chromatography on Biogel A-5m/0.5m revealed essentially the presence of HM-, α-, β- and γ-crystallin. A comparison of the various elution patterns showed the presence of relatively small amounts of HM-crystallin in the nuclear part of the cataractous lenses. A remarkable feature of human γ-crystallin was its range of isoelectric points, namely between pH 5 and 7, deviating from that of γ-crystallin from other mammals (cow and rabbit), which lies between pH 7 and 8. Furthermore, it appeared that part of the β- and γ-crystallin polypeptide chains are identical. This finding, as well as immunochemical evidence, implies that non-aggregated β-crystallin chains are present in the γ-crystallin fraction. The US fraction appeared to be composed of α-, β- and γ-crystallin subunits and a considerable amount of acidic chains in the pH 4.7 to 5.4 range.

In this study it became clear that marked changes in rabbit lens proteins appeared abruptly at the mature stage of the X-ray induced cataract. Essentially three types of events have taken place:

i. aggregation and insolubilization of crystallins,
ii. selective disappearance of soluble protein,
iii. deamidation and partial degradation.

The aggregation and insolubilization turned out to involve all the crystallins, although α-crystallin was the main component. The crystallins which disappeared, must have leaked out of the lens. Leakage may be caused by a change in permeability of the lens capsule, as a result of the swelling phenomenon during cataract development. In any case, a considerable damage of the fibre cell membranes was demonstrated. Another possibility is that protein molecules are first dissociated into subunits before leaking out, since from data in literature it is known that bovine lens capsule is permeable to molecules with the size of γ-crystallin. Still another possibility is proteolysis, which may of course be involved in the leakage process.

A comparison of the results found for the cataractous lenses from rabbit and man revealed:

- for both the presence of HM-crystallin, which electron microscopically showed the same type of aggregates.

- for both the occurrence of great amounts of US fractions, with a subunit composition consisting of α-, β- and γ-crystallin polypeptide chains.
- the presence of acidic polypeptide chains with isoelectric points between pH 4.7 and 5.4 in great amounts in the US fractions of human lens preparations, in contrast with those of the rabbits. This difference between rabbit and man may be attributed to differences in age; in the case of the model system, the cataract was formed at a relatively young age, whereas senile cataract in man occurred in older age.
- a complication for further research, because the isoelectric points of the human γ-crystallins do not show the characteristic high values as found for the rabbit, and because β-crystallin polypeptide chains seem to be present in the γ-crystallin fraction.

In conclusion, from the present study distinct indications were obtained that processes in cataract are essentially progressive aging processes, as far as it concerns the lens proteins. Nevertheless, the analogy is not complete. A broader basis should be laid from which the remaining differences between the processes of aging and cataract formation can be explained.

Het doel van het in dit proefschrift beschreven onderzoek was de bestudering van mogelijke veranderingen in de verdeling en de subeenheidstructuur van ooglenseiwitten als gevolg van staarvorming Als experimenteel model werd éenzijdige Rontgen-cataract bij het konijn gekozen

Een eerste vereiste is gedegen kennis van de eigenschappen en kenmerken van de normale ooglenseiwitten. Een karakterisering van de crystallines uit de ooglens van het konijn is in hoofdstuk 2 te vinden.

Naast één α-, drie β- en een γ-crystalline fractie werd een eiwitfractie met zeer hoog moleculair gewicht (HM-crystalline) waargenomen. Dit HM-crystalline vertoonde een subeenheidstructuur, overeenkomend met die van het α-crystalline

Literatuurgegevens maakten aannemelijk, dat de water-onoplosbare fractie (albumoid), die onderverdeeld kan worden in een ureum-oplosbare (US) fractie en een ureum-onoplosbare (UI), uit membraanfragmenten bestaande, fractie, ontstaan is uit α-crystalline. Het was ook bekend, dat het albumoid in cataracteuze lenzen in grote hoeveelheden wordt aangetroffen. Teneinde de relatie tussen albumoid en α-crystalline te verifieren werd de polypeptide-ketensamenstelling van het α-crystalline en die van de US-fractie onderzocht (hoofdstuk 3) Als uitgangsmateriaal werd de buitenste lenscortex (jonge cellen, 40% van het totale natte gewicht van de lens) en de lensnucleus (oude cellen, 20% van het natte gewicht) gebruikt De verkregen resultaten toonden aan, dat corticaal α-crystalline een betrekkelijk eenvoudige, nucleair α-crystalline daarentegen een complexere peptideketenstructuur heeft Deze complexiteit lijkt ten dele veroorzaakt te zijn door deamidatie- en partiele degradatie-processen, die samenhangen met de leeftijd. Daarnaast is het complexe karakter te wijten aan de aanwezigheid van kleinere hoeveelheden polypeptideketens van het β-crystalline De peptidepatronen van het HM-crystalline en van de US-fractie uit cortex en kern kwamen overeen met die van de overeenkomstige α-crystallines Op basis van deze gelijke samenstelling werd aangenomen, dat HM-crystalline een tussenproduct is bij het onoplosbaar worden van crystallines Verdere steun voor deze aanname was het feit, dat door middel van elektronenmicroscopie bij HM-crystalline een populatie van aggregaten met deeltjesgrootte tot 500 nm werd waargenomen (hoofdstuk 4) Nader onderzoek van HM-crystalline door middel van ionenwisselaarchromatografie bracht aan het licht, dat deze zeer grote eiwitaggregaten konden worden gedissocieerd tot en gescheiden in α- en β-crystallines met sedimentatiecoefficienten van respectievelijk 20 S en 4 S Uit dit gegeven vloeiden bepaalde voorstellingen betreffende de rangschikking van de α- en β-crystallines in het HM-crystalline voort.

In hoofdstuk 5 wordt de subeenheidsamenstelling beschreven van α-crystalline, HM-crystalline en de US-fractie, verkregen uit de door Rontgen-straling geinduceerde, rijpe cataract De verkregen resultaten toonden aan, dat de peptideketensamenstelling complexer werd in genoemde volgorde en overeenkwam met die van de corresponderende fracties uit de normale lenskern De drie fracties bestonden uit α- en - in successievelijk toenemende hoeveelheden - β-crystallineketens, in de US-fracties kwamen bovendien geringe hoeveelheden γ-crystallineketens voor Tussen cortex en nucleus was er in dit opzicht geen verschil Het was opvallend, dat HM-crystalline uit de cataracteuze cortex reeds het complexe patroon vertoonde, dat normaliter slechts in de kern aangetroffen werd Bovendien bleek het HM-crystallinegehalte in de cataracteuze cor-

tex zeer hoog te zijn, hetgeen wederom slechts in de nucleus van de controlelens werd gevonden. Elektronenmicroscopisch vertoonde HM-crystalline uit de cataracteuze cortex de aanwezigheid van zeer grote aggregaten, zulks in tegenstelling tot HM-crystalline uit de normale cortex, dat betrekkelijk kleine aggregaten liet zien. Deze resultaten suggereerden alle, dat in lenzen met een rijpe cataract een progressief verlopend verouderingsproces heeft plaats gevonden.

Hoofdstuk 6 is gewijd aan het verloop van de aggregatie en het onoplosbaar worden van de crystallines na de Röntgen-bestraling, te weten één, twee en drie maanden na de bestraling. Duidelijke veranderingen openbaarden zich pas drie maanden na de bestraling (rijpe cataract). Het HM-crystallinegehalte was vooral in de cortex zeer sterk toegenomen. De US-fractie vertoonde ten opzichte van de controle een tienvoudige toename. Bovendien vond tussen de tweede en derde maand na de bestraling een afname van het totaal eiwitgehalte plaats: van de β-crystallines verdween 55%, van het γ-crystalline 65%.

Met de leeftijd samenhangende, post-synthetische processen bleken in het α-crystalline van de controlelens te hebben plaats gevonden, hetgeen tot uiting kwam in veranderde hoeveelheden gedeamideerde en verkorte polypeptideketens. Het α-crystalline uit cortex van de bestraalde lens vertoonde een subeenheidstructuur overeenkomstig met die van de kernpreparaten. Deze ogenschijnlijk vroegtijdige veroudering van α-crystalline in jonge cellen en de extreme toename van de hoeveelheid albumoid vormden verdere aanwijzingen, dat bij Röntgen-cataract processen hebben plaats gevonden die op versnelde veroudering lijken.

In hoofdstuk 7 zijn de karakteristieken van de crystallines uit oude normale en seniele cataracteuze menselijke ooglenzen beschreven. Fractionering van het oplosbaar eiwit door middel van gelchromatografie op Biogel A-5m/0.5m leverde in wezen HM-, α, β- en γ-crystalline op. In de cataracteuze lenskern bleek relatief weinig HM-crystalline aanwezig te zijn. Bij de humane γ-crystallines was het bereik van de isoelectrische punten opmerkelijk; het lag tussen pH 5 en 7 en week daarmee duidelijk af van het bereik bij andere zoogdieren (koe en konijn), dat tussen pH 7 en 8 ligt. Verder werd waarschijnlijk, dat de polypeptideketens van het β- en γ-crystalline ten dele identiek zijn. Deze bevinding, evenals immunochemische aanwijzingen, wees erop, dat β-crystallineketens als zodanig in de γ-crystallinefractie aanwezig zijn. De US-fractie bleek te zijn samengesteld uit de subeenheden van α, β- en γ-crystalline alsmede een aanzienlijke hoeveelheid opvallend zure ketens met isoelectrische punten van 4.7 tot 5.4.

Uit het onderzoek is duidelijk geworden, dat uitgesproken veranderingen in de structuur van de lenseiwitten vrij abrupt verschijnen bij het bereiken van de rijpe fase van de door Röntgen-stralen geinduceerde cataract bij het konijn. In wezen traden drie typen van processen op:

1. aggregatie en onoplosbaar worden van crystallines,
2. selectief verlies van oplosbaar eiwit,
3. deamidatie en verkorting van polypeptideketens.

Er is gebleken, dat alle crystallines betrokken zijn bij de aggregatie en precipitatie van lenseiwitten, zij het dat α-crystalline hierbij de voornaamste component is. De verdwenen crystallines moeten uit de lens zijn gelekt. Deze lekkage kan bij voorbeeld veroorzaakt worden door verhoogde permeabiliteit van de lenskapsel als gevolg van zwelling gedurende de cataractontwikkeling; aanzienlijke schade aan de vezelcelmembranen kon in ieder geval worden aangetoond. Een andere mogelijkheid zou zijn, dat de eiwitmoleculen, na eventuele dissociatie tot subeenheden, uit de lens lekken, aangezien

uit de literatuur bekend is, dat het kapsel van de koelens permeabel is voor moleculen ter grootte van γ-crystalline Nog een andere veronderstelling is uiteraard proteolyse, die voorafgaand aan het lekkageproces een rol kan spelen.

Een vergelijking van de resultaten, die bij de cataracteuze lenzen van konijn en mens werden gevonden, toonde het volgende aan.

- Voor beide de aanwezigheid van zeer hoogmoleculaire crystallines, die elektronenmicroscopisch hetzelfde type van eiwitaggregaten vertoonden
- Voor beide het voorkomen van grote hoeveelheden US-fractie met een subeenheidstructuur, waarin de polypeptideketens van α-, β- en γ-crystalline vertegenwoordigd zijn.
- De aanwezigheid van zure polypeptideketens met isoelectrische punten tussen 4 7 en 5.4 in grote hoeveelheden in de US-fractie van de menselijke lens, zulks in tegenstelling tot die van het konijn Deze verschillen tussen mens en konijn zijn mogelijk te wijten aan verschillen in leeftijd, bij het modelsysteem werd de cataract op relatief jonge leeftijd verwekt, terwijl de seniele cataract bij de mens pas op latere leeftijd voorkomt.
- Een complicatie voor verder onderzoek, omdat de isoelectrische punten van humaan γ-crystalline niet de karakteristieke, hoge waarden hadden als die bij het konijn en omdat polypeptideketens van het β-crystalline aanwezig lijken te zijn in de menselijke γ-crystallinefractie

Uit het onderzoek volgt verder, dat er duidelijke aanwijzingen zijn voor de veronderstelling, dat het ontstaan van cataract in wezen een progressief verlopend verouderingsproces is voor zover het de lenseiwitten betreft De analogie is echter niet volledig. Er moet een bredere basis worden gevonden, van waaruit de aanwezige verschillen kunnen worden verklaard.

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Curriculum vitae

De auteur, mevrouw K.N. Liem-The werd op 24 juli 1940 te Soerabaya, Indonesië geboren. Te Soerabaya en Rotterdam werd de lagere school tot 1952 bezocht. Na het behalen van het diploma S.M.A.-B aan de Katholieke Middelbare School "Sancta Ursula" te Jakarta in 1958 begon zij met de studie in de Farmacie aan de Rijksuniversiteit "I.T.B." te Bandoeng. Het candidaatsexamen werd afgelegd in juni 1962 en het doctoraalexamen in augustus 1967. In mei 1968 vertrok zij samen met haar echtgenoot, P.L. Liem, naar Nederland. Van 1 januari 1971 tot 1 januari 1975 was zij in dienst van de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek (Z.W.O.). Als wetenschappelijk ambtenaar aan het laboratorium voor Biochemie van de Katholieke Universiteit te Nijmegen, heeft zij onder leiding van Dr. H.J. Hoenders en Prof. Dr. H. Bloemendal het hier beschreven onderzoek verricht.

STELLINGEN

I

Bij de isolering van crystallines is het aan te bevelen om als eerste methode gebruik te maken van moleculaire zeven in plaats van ionenwisselaars.

II

Het woord "hyalin bodies" in leverbiopten is een betere, meer algemene naamgeving dan de term "Mallory bodies".

III

De door Bonnell en Selander getrokken conclusie dat bij zeeolifanten een uniforme homozygotie van de door hen genoemde loci aanwezig zou zijn, is onjuist.

M.L. Bonnell en R.K. Selander, Science **184**, 908-909 (1974)

IV

Het is onwaarschijnlijk dat de "fractie f" in koninginnegelei van de honingbij (Royal jelly) een verjongende werking heeft op de menselijke huid.

V

De door Satoh verstrekte gegevens over de verdeling van lenseiwitten in de humane lens, kunnen slechts zeer globaal worden beschouwd.

K. Satoh, Exp. Eye Res. **14**, 53-57 (1972)

VI

De geleidelijke atrofie van de hepatocyten gepaard gaande met ductulaire proliferaties in de lever van de gastheer is het best te bestuderen in het model van de heterotype, auxiliare levertransplantatie volgens Hess.

F. Hess, C. Jerusalem en M.N. van der Heyde, Arch. Surg. **104**, 76-80 (1972)

VII

De verschillen in heterogeniteit tussen de 40 S ribosomale subunits van tumor ascites cellen groeiend in vivo respectievelijk in vitro kunnen herleid worden tot de verschillen in fysiologische condities van deze cellen.

M. Sameshima en M. Izawa, Biochim. Biophys. Acta 378, 405-414 (1975)

VIII

De tabel van Darnall en Klotz over de subunits van eiwitten verliest aan waarde door de soms zeer oude referenties.

D.W. Darnall en I.M. Klotz, Arch. Biochim. Biophys. 166, 651-682 (1975)

IX

Het moet worden betwijfeld, of de intracellulaire pH of electrolyt-concentratie in de pancreas zoals bepaald door Swanson en Solomon met behulp van analyses op het gehele weefsel, inderdaad van toepassing zijn op de ductulaire cellen, omdat die slechts een klein deel van het totale weefselvolume innemen.

C.H. Swanson en A.K. Solomon, J. Gen. Physiol. 62, 407-429 (1973)
C.H. Swanson en A.K. Solomon, J. Gen. Physiol. 65, 22-45 (1975)

X

Lasser en Balasz wekken de indruk dat alle membranen in de lens van het tight junction type zijn. Dit wordt weersproken door verschillende resultaten, zowel van henzelf als van anderen.

A. Lasser en E.A. Balasz, Exp. Eye Res. 13, 292-308 (1972)
I. Dunia, C.S. Ghosh, E.L. Benedetti, A. Zweers en H. Bloemendal, FEBS Letters, 45, 139-144 (1974)

Nijmegen, 17 april 1975 K.N.Liem-The